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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

HORST LINDHOFER et al.

Application No.: 09/094,921

Filed: June 15, 1998

For: METHOD FOR EX VIVO IMMUNIZATION USING HETEROLOGOUS INTACT BISPECIFIC AND/OR TRISPECIFIC ANTIBODIES

Examiner: Holleran, A.

Art Unit: 1642

DECLARATION UNDER 37 CFR 1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

I, HORST LINDHOFER, hereby declare as follows:

1. I am one of the inventors named in the above-referenced patent application.

2. I am one of the authors of each of the three published papers attached hereto as Exhibits, whose Exhibit Nos. and citations are listed below:

- Zeidler, R., et al., "Simultaneous Activation of T Cells and Accessory Cells by a New Class of Intact Bispecific Antibody Results in Efficient Tumor Cell Killing," *J. Immunol.*163:1246-1252 (1999)

Zeidler, R., et al., "The Fc-region of a new class of intact bispecific antibody mediates activation of accessory cells and NK cells and induces direct phagocytosis of tumour cells," *Brit. J. Cancer* 83(2):261-266 (2000)

Ruf, P., et al., "Induction of a long-lasting antitumor immunity by a trifunctional bispecific antibody," *Blood* 98(8): 2526-2533 (15 October 2001)

3. The experiments reported in each of these papers were performed either by me, under my supervision or direction, or by a research team of which I was a part. The descriptions of the procedures of each experiment and the results obtained therefrom as presented in these papers, which I have personally reviewed, are accurate and truthful reports of experiments actually performed.

4. I further declare that all statements herein that are made of my own knowledge are true and that all statements herein that are made on information and belief are believed to be true, and that all such statements are made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both (18 U.S.C. 1001) and may jeopardize the validity of the application or any patent issuing thereon.

Horst Lindhofer Date: 7th of March 2002

Horst Lindhofer

MHH
SF 1323022 v1

Simultaneous Activation of T Cells and Accessory Cells by a New Class of Intact Bispecific Antibody Results in Efficient Tumor Cell Killing[1]

Reinhard Zeidler,* Gilbert Reisbach,† Barbara Wollenberg,* Stephan Lang,* Sarita Chaubal,* Bärbel Schmitt,* and Horst Lindhofer[2]*

Bispecific Abs (bsAb) are promising immunological tools for the elimination of tumor cells in minimal residual disease situations. In principle, they target an Ag on tumor cells and recruit one class of effector cell. Because immune reactions in vivo are more complex and are mediated by different classes of effector cell, we argue that conventional bsAb might not yield optimal immune responses at the tumor site. We therefore constructed a bsAb that combines the two potent effector subclasses mouse IgG2a and rat IgG2b. This bispecific molecule not only recruits T cells via its one binding arm, but simultaneously activates FcγR$^+$ accessory cells via its Fc region. We demonstrate here that the activation of both T lymphocytes and accessory cells leads to production of immunomodulating cytokines like IL-1β, IL-2, IL-6, IL-12, and DC-CK1. Thus this new class of bsAb elicits excellent antitumor activity in vitro even without the addition of exogenous IL-2, and therefore represents a totally self-supporting system. ***The Journal of Immunology,*** **1999, 163: 1246–1252.**

Successful immune responses against neoplastic cells in vivo depend on the cooperation of different classes of immune cells. Malignant cells may be targets for cytotoxic T cells that recognize specific MHC/peptide complexes on the cell surface or they may be eliminated by NK cells or monocytes/macrophages. However, these different classes of effector cell depend on each other with respect to the production of cytokines and the delivery of costimulatory signals and, therefore, they usually operate in a concerted manner. T cells are thought to be the most important subpopulation for killing of neoplastic cells. But full activation of naive T cells depends on proper Ag presentation by professional APCs or activated accessory cells and costimulatory molecules like CD40, LFA-3, B7.1, and B7.2 in the presence of cytokines such as IL-2 and IL-12 (1, 2). Tumor cells normally do not express B7 molecules, and instead of activating specific T cells can even cause their anergy (3, 4).

Bispecific Abs (bsAb)[3] are regarded as powerful tools for the immunological treatment of malignant cells in a minimal residual disease situation, because single disseminated tumor cells are especially appropriate targets for an immunological attack. However, the bsAb described to date normally activate only a single class of effector cell, i.e., either T cells, NK cells, FcγRI$^+$, or FcαRI$^+$ cells (5–7) following binding to an appropriate target molecule of the effector cell. Here we show data obtained with a new class of bsAb consisting of the two potent and evolutionary related effector subclasses, mouse IgG2a and rat IgG2b. This intact bsAb (BiUII) is able to simultaneously activate T cells (via one arm) and accessory cells (via the Fc region) in the vicinity of tumor cells. In contrast to a similar T cell-redirecting bsAb, SHR-1, with the subclass combination mouse IgG1 × rat IgG2b (8), BiUII does not depend on the addition of exogenous IL-2 to provide full antitumor activity. This reveals the importance of the subclass combination for induction of activation signals via the Fc region of accessory cells. Moreover, we demonstrate that the antitumor efficiency of our bsAb is strongly reduced when T cells or accessory cells alone are used as effector cells. We therefore postulate that a "Tri-cell-complex" consisting of tumor cells, T lymphocytes, and accessory cells is created by this new class of bsAb. Only the formation of this complex results in a full activation of different effector cells providing optimal antitumor efficiency.

Materials and Methods

Cell lines and PBMC preparation

Fadu (American Type Culture Collection, Manassas, VA) and PCI-1 are Ep-CAM-positive established squamous carcinoma cell lines of the head and neck (SCCHN) and were maintained in DMEM with 10% FCS. Both cell lines express epithelial cell adhesion molecule (Ep-CAM) and MHC class I, but not MHC class II as tested by flow cytometry (data not shown). DG75 is an EBV-negative Burkitt lymphoma cell line. PBMC were isolated from heparinized blood of voluntary donors by Ficoll density centrifugation. Where indicated (=PBL), the monocyte/macrophage fraction was removed by adhesion to plastic flasks twice for 2 h at 37°C in an incubator.

Monoclonal Abs

The following hybridomas have been used: 26116 (rat IgG2b, anti-CD3; kindly provided by R. Schuh, Gesellschaft für Strahlung und Umweltforschung (GSF), Munich, Germany) and C215 (mouse IgG2a, anti-Ep-CAM; kindly provided by M. Dohlsten, Pharmacia Upjohn, Uppsala, Sweden).

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[3] Abbreviations used in this paper: bsAb, bispecific Abs; SCCHN, squamous carcinoma cell lines of the head and neck; DC, dendritic cells; ADCC, Ab-dependent cellular cytotoxicity; Ep-CAM, epithelial cell adhesion molecule.

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Table I. *PCR primers used for the amplification of cytokines*

Cytokine	Upstream	Downstream
IL-1β	cgatcactgaactgcacgctccg	ggtgaagtcagttatatcctggccg
IL-2	caagaatcccaaactcaccagg	caatggttgctgtctcatcagc
IL-4	cggacacaagtgcgatatcacc	ccaacgtactctggttggcttcc
IL-6	cacacagacagccactcacctc	ctcaggctggactgcaggaac
IL-12 (p40)	atgccctgtacttgagtttcaacc	gttagcccccctcagtaatgttcttc
DC-CK1	tgggtccagaaatacatcagcg	acgaagagttgaagggaaaggg
GAPDH	aattccatggcaccgtcaag	gcctgcttcaccaccttctt

RT-PCR

Total RNA from primary lymphocytes was isolated after up to 72 h of incubation with allogeneic SCCHN. The RNA preparations were treated with RNase-free DNase (Boehringer Mannheim, Mannheim, Germany) for 30 min at 37°C. After inactivation of the enzyme, 1 μg RNA was reverse transcribed (Superscript Plus, Life Technologies, Gaithersburg, MD) with an oligo(dT) primer for 60 min at 42°C. PCR with one-tenth of the volume (2 μl) was performed in a buffer containing 1.5 mM $MgCl_2$, 100 pmol of each primer, 0.2 mM final concentration of each dNTP, and 0.5 μl Goldstar *Taq* polymerase (Eurogentec, Seraing, Belgium) in a final volume of 50 μl in an Perkin-Elmer (Norwalk, CT) thermal cycler. PCR primers are shown in Table I. Amplified bands were analyzed by electrophoresis in a 1.5% agarose gel and by ethidium bromide staining.

Generation of dendritic cells (DC)

The adherent fraction of PBMC was incubated for 7 days in Iscove's medium with 5% FCS (Life Technologies) and 800 U/ml of each human IL-4 and GM-CSF (both Boehringer Mannheim).

FACS analysis

For FACS analysis, 10^5 cells were incubated with the primary Ab for 30 min on ice in PBS/5% FCS. The cells were washed twice in PBS and incubated for another 30 min with the second FITC-labeled Ab. After two final washings, propidiumiodide was added, and flow cytometry was performed using a FACSCalibur cytometer and the CellQuest analysis program (Becton Dickinson, Heidelberg, Germany). For isolation of highly purified $CD2^+$ cells, PBMC were incubated with FITC-labeled Abs (PharMingen, Hamburg, Germany) and separated on a FACSCalibur.

Production of BiUII

The BiUII quadroma was produced as previously described (9). To isolate hybrid Ab molecules of the subclass combination rat IgG2b/mouse IgG2a from hybridoma supernatants, the supernatants were centrifuged, filtered, and loaded onto a 5 ml Econo Pac protein A column (Bio-Rad, Richmond, CA). After washing with 10 volumes of PBS, Ab with the hybrid heavy chain configuration was eluted with 0.1 M citric acid (pH 5.1).

Cell culture and killing efficiency

For determination of bsAb-mediated killing of tumor cells and cytokine production, 1×10^4/well SCCHN (targets, =T) were pipetted in 96-well flat-bottom plates (Falcon, Franklin Lakes, NJ), and PBMC or subpopulations of these effectors (=E) were added at ratios from 40:1 to 1:1 E:T. bsAb was used at 10 ng/well in a total volume of 100 μl/well RPMI with 10% FCS. Plates were incubated for 3 days at 37°C in a humidified atmosphere and 5% CO_2. All SCCHN were proven to express Ep-CAM and to lack costimulatory molecules CD80 and CD86.

MTT assay

To assess bsAb-mediated tumor cell killing, a colorimetric MTT-based assay was performed as described previously (10). Briefly, SCCHN target cells were plated in wells of a 96-well flat-bottom plate and incubated overnight to prepare semiconfluent cell monolayers. Effector cells were added to the tumor cell monolayers at the appropriate ratios, and plates were incubated for 24–48 h. After removing effector cells by washing, MTT solution (0.5 mg/ml; Sigma, Deisenhofen, Germany) was added, and plates were incubated for a further 4 h. The MTT solution was removed, and blue crystals of formazan formed in viable tumor cells were dissolved by adding DMSO. Plates were read at 540 nm in a spectrophotometer, and the results were calculated based on the mean of absorbance obtained from at least six wells according to the following formula: % cell death = 100 × $(C - E)/(C - B)$, where C is the optical density reading of the cells with target cells in the absence of effectors (control), B is background without any cell population, and E is the optical density reading of adherent tumor cells remaining in the wells after coincubation with effector cells.

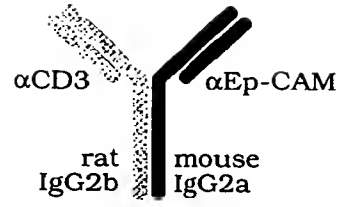

FIGURE 1. Composition of the intact bsAb BiUII. BiUII is a chimeric molecule consisting of a rat IgG2b chain that is specific for human CD3 and a mouse IgG2a chain which targets the human pan-carcinoma Ag, Ep-CAM (also known as 17-1A and GA733-2 Ag), (35, 36).

Bioassays

Biologically active levels of IL-2, IL-6, TNF, and GM-CSF were measured in 96-well flat-bottom microtiter plates using cytokine dependent growth of cell lines CTLL-2, 7TD1, WEHI-164, and TF-1, respectively. Cell culture supernatants were titrated in duplicates and diluted from 1:5 to 1:10000. Standards of recombinant human IL-2 (PBH, Hannover, Germany), human IL-6 (Boehringer Mannheim), human TNF-α (PBH), or human GM-CSF (Boehringer Mannheim) were included in each assay to generate a standard curve. Intra- or interassay variability was less than 10% or 20%, respectively. Specificity of bioassays was confirmed by neutralizing active samples with cytokine specific Abs (PBH and Boehringer Mannheim). The lower detection limits of IL-2, IL-6, TNF, and GM-CSF were 20 pg/ml, 10 pg/ml, 0.1 ng/ml, and 10 pg/ml, respectively.

Results

Construction of BiUII

Ep-CAM is an Ag that is overexpressed on many carcinomas of different origin (11). Therefore, it was selected as a target tumor molecule for our bsAb. Because T cells are believed to be the most important effector cells for tumor cell elimination, they were targeted for activation via CD3. The bsAb constructed to recognize these two Ags was designated BiUII. Because most tumor cells do not express costimulatory molecules, BiUII was constructed with mAbs of subclasses that bind and activate human $Fc\gamma RI^+$ cells (12). The quadroma that resulted from the fusion of the anti-Ep-CAM and the anti-CD3 hybridomas was characterized and intact bsAb was purified as described (9). As shown in Fig. 1, BiUII represents a chimeric molecule consisting of the evolutionary related and highly homologous mouse IgG2a and rat IgG2b heavy chains.

Lysis capacity of BiUII

To determine the antitumor efficiency of BiUII, we first evaluated its capacity to mediate the killing of the Ep-CAM-positive tumor cell line PCI-1 (13) and compared it with the two monoclonal parental Abs (αCD3 and αEp-CAM). PCI-1 cells were cocultivated with PBMC in the presence of either BiUII, both parental Abs, or for control purposes, without Ab. After 2 days of culture, the numbers of remaining tumor cells were determined in a standard MTT assay. As shown in Fig. 2, BiUII displayed a much higher lytic capacity for tumor cells than equimolar concentrations of the corresponding parental Abs. Even 10-fold higher concentrations (50 ng/100 μl) of these Abs were less efficient than BiUII (data not shown). Theoretically, the two parental Abs also recruit $Fc\gamma R^+$ cells and activate T cells via CD3. However, their observed antitumor capacity was much lower. This result suggested that BiUII-mediated formation of a complex involving at least two different classes of immune cells.

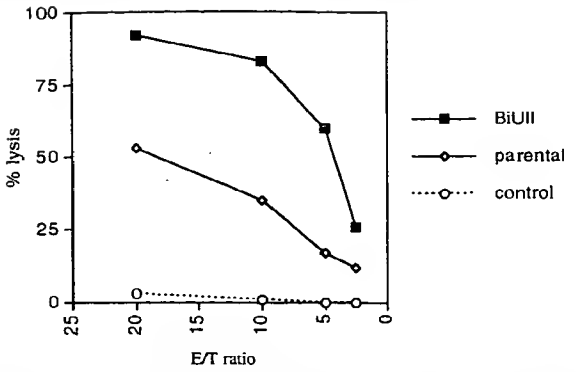

FIGURE 2. Lysis of PCI-1 cells. PBMCs from a healthy volunteer were incubated with PCI-1 in the presence of BiUII (5 ng/100 μl), both parental Abs (5 ng/100 μl each), or without Abs for 2 days. Regarding the lytic capacity, BiUII displays a much higher lytic activity compared with the parental Abs that were used simultaneously. One of five representative experiments is shown.

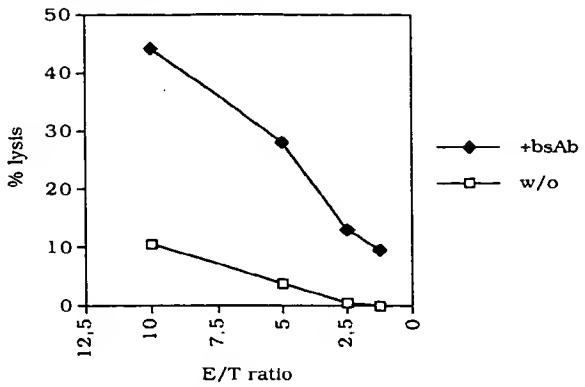

FIGURE 3. Killing of PCI-1 cells by monocytes and DC. Adherent cells derived from the peripheral blood of a voluntary donor were isolated by plastic adhesion and incubated for 2 days with PCI-1 with (50 ng/ml) or without bsAb. T cells were not detectable in the cell preparation. Killing of PCI-1 was determined in a MTT assay.

The lytic capacity of BiUII was not further enhanced by the addition of exogenous IL-2 (100 U/ml; data not shown). This finding contrasts with results described recently using an intact Ab composed of the subclasses mouse IgG1 × rat IgG2b, which showed enhanced activity in the presence of exogenous IL-2 (8).

Induction of ADCC by BiUII

Ab-dependent cellular cytotoxicity (ADCC) is considered to be one of the most effective mechanisms for the destruction of virally infected cells and tumor cells (14). Both macrophages and DC express the high affinity FcγRI (CD64) (15) and can be stimulated to ADCC by particular subclasses of opsonizing IgG Abs. Because BiUII is a chimeric molecule consisting of a rat IgG2b chain and a murine IgG2a chain (Fig. 1), it can theoretically bind and activate FcγRI$^+$ cells (12), a property that may contribute to BiUII-mediated tumor cell killing. It has been demonstrated recently that the unconjugated anti-Ep-CAM 17–1A mAb has a therapeutic effect that is probably due to ADCC (16, 17). To determine whether BiUII could induce ADCC, we isolated macrophages and DC from peripheral blood by plastic adhesion and cocultured them with PCI-1 target cells with or without BiUII. The induction of ADCC measured in a MTT assay clearly demonstrated that PCI-1 cells were lysed much more efficiently in the presence of BiUII (Fig. 3). Because T lymphocytes were not present in this assay, as determined by FACS analysis (data not shown), the lysis of PCI-1 cells was most likely due to BiUII-mediated ADCC.

Accessory cells are necessary for optimal antitumor activity

Because BiUII targets T lymphocytes and FcγRI$^+$ cells, both of which are activated by Ab binding, we questioned whether accessory cells contributed to the BiUII-mediated antitumor activity for example via direct phagocytosis. Therefore, we compared the killing capacity of unseparated PBMCs with highly purified T cell populations. CD2$^+$ T cells were isolated by cell sorting and used in a MTT assay. We compared this purified T cell population with whole PBMC for killing activity directed against PCI-1 cells. As shown in Fig. 4, maximal antitumor activity was achieved when PBMCs were used as the effector source of cells. This result substantiated the importance of accessory cells targeted via their Fc receptors in tumor cell killing.

Induction of cytokine production

Stimulation of T lymphocytes leads to IL-2 production and upregulation of CD25, the α-chain of the IL-2R. IL-2 is the most important autocrine growth factor for T cells (18). In addition, activated T cells induce IL-12 production by monocytes/macrophages and DC (19), thereby enhancing antitumor activity (20).

The high affinity FcγRI (CD64) is expressed by monocytes/macrophages and dendritic cells (15). Because BiUII combines mouse IgG2a and rat IgG2b subclasses, it should theoretically be able to bind and activate FcγRI$^+$ cells (12). Therefore, tumor cells opsonized by BiUII are complexed to accessory cells via CD64 and ADCC may be induced via direct phagocytosis. Subsequently, phagocytosed tumor-derived proteins can be processed and presented by MHC class I and II molecules, leading to humoral and cellular immunity. In addition, activated accessory cells can deliver cytokines like IL-6 and costimulatory signals via molecules like CD40, LFA-3, CD80, and CD86 that are mandatory for T cell activation and prevention of anergy (21).

Therefore, we determined whether BiUII was able to induce the production of IL-2 and IL-6 in PBMCs when cocultured in the presence of PCI-1 cells, thereby documenting the activation of both T cells and accessory cells. As shown in Fig. 5, PBMCs produce both cytokines only when BiUII is present. IL-2, which is mainly T cell derived, is produced in essentially equal amounts using either PBMCs or PBLs as effector cells. In contrast, IL-6 is

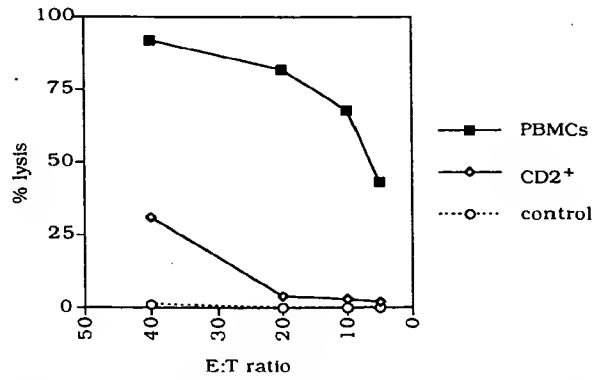

FIGURE 4. Only whole PBMC display full antitumor activity in the presence of BiUII (50 ng/ml). MTT assays with whole PBMCs and CD2$^+$ T cells were performed, and the lysis of PCI-1 cells was determined. PBMC that contain FcγRI$^+$ accessory cells show a much higher capacity for tumor cell killing than purified T cells, especially at lower E:T ratios. The results of one of three representative experiment are shown.

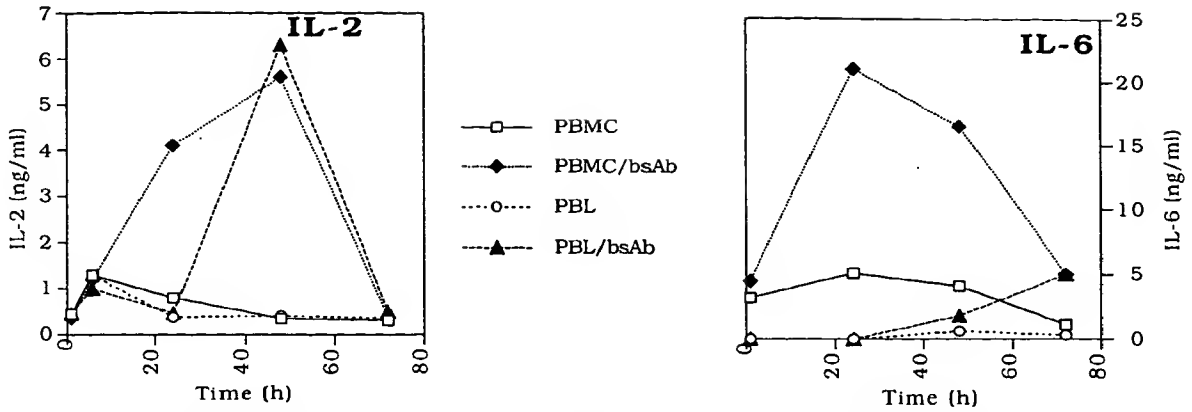

FIGURE 5. . Production of biologically active IL-2 and IL-6 by PBMCs and PBLs. Peripheral blood cells were coincubated with PCI-1 cells in the presence or absence of BiUII. Cytokine levels in the supernatants were determined by proliferation bioassays. Whereas the T cell-derived IL-2 is produced at almost identical concentrations by both PBMC and PBL, the levels for IL-6 that is secreted mainly by activated monocytes/macrophages is much higher when PBMC are used as effectors. The initial IL-6 concentrations in PBMC ± bsAb are due to the activation of monocytes/macrophages during isolation.

mainly produced by activated monocytes/macrophages and was only detectable when PBMCs were used but was not present when the adherent fraction was depleted. This finding supports our contention that the Fc region of BiUII mediates activation of $Fc\gamma RI^+$ cells.

We also compared the bsAb with the two parental Abs for the production of IL-1β, IL-2, IL-4, IL-6, and IL-12. As depicted in Fig. 6, cytokine levels were only slightly elevated compared with background (IL-2 and IL-12), or were not induced at all (IL-1β, IL-4, and IL-6) when the parental Abs were used as stimulators. In contrast, after incubation with the bsAb, we observed a clear induction of all cytokines examined. These data and the fact that BiUII is much more efficient in tumor cell killing than the parental Abs suggest that the local production of various cytokines is likely a prerequisite for efficient tumor cell killing.

BiUII-mediated production of IL-2 depends on the presence of the target molecule, Ep-CAM

The major drawback in the in vivo application of bsAb is the problem of side effects provoked by uncontrolled cytokine release. In particular, the nonspecific activation of T cells via CD3 is thought to produce elevated IL-2 levels that can cause severe side effects. To evaluate IL-2 induction by BiUII, PBMCs were incubated with the Burkitt lymphoma cell line DG75 that does not express Ep-CAM, or with a subclone of DG75 that was stably transfected with an expression plasmid for Ep-CAM (DG75/Ep-CAM). As shown in Fig. 7, significant concentrations of IL-2 were only produced when PBMC were cultivated with DG75/Ep-CAM but not with DG75 cells. This finding implies that full activation of T cells and production of IL-2 is only achieved as part of a Tri-cell-complex involving accessory cells and tumor cells. A dependence on the presence of tumor cells expressing the target Ag Ep-CAM is an important parameter that can limit the risk of uncontrolled systemic IL-2 production by this bispecific reagent.

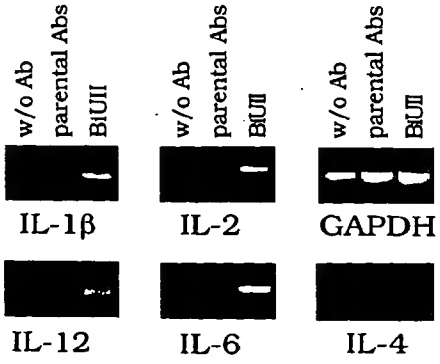

FIGURE 6. Cytokine production is induced by BiUII but not by a combination of the two parental Abs. The induced expression of IL-1β, IL-2, IL-4, IL-6, and IL-12 (p40) is in accordance with the higher antitumor activity of BiUII and demonstrates the superiority of this new class of bsAb in tumor cell killing.

Activation of DC

DC are key regulators of immune responses. They form a system of efficient Ag presenting cells which present Ags to T cells (22) and trigger their activation via the CD40 dependent pathway (23). Recently, a DC-specific cytokine, DC-CK1, has been identified that is exclusively expressed by DC at high levels at sites of immune responses (24). DC-CK1 elicits a profound chemotactic activity to attract $CD45RA^+$ naive T cells. Thus it is believed that DC are directly involved in the generation of cytolytic T lymphocytes (23, 25).

Because DC have recently been shown to express CD64 (15), we wanted to determine whether peripheral blood DC contribute to BiUII-mediated killing of tumor cells. The adherent fraction of PBMCs was incubated for 7 days in the presence of IL-4 and GM-CSF and then cocultured with PCI-1 cells with or without BiUII. Total RNA was then isolated at two different time points. Whereas after 4.5 h of incubation no substantial differences in DC-CK1 levels were detectable, the expression of the cytokine was clearly higher in the presence of BiUII 16 h later, indicating bsAb-mediated activation of DC (Fig. 8).

Discussion

We describe here the antitumor properties of a new class of bsAb. To our knowledge, this is the first investigation that demonstrates the simultaneous activation in vitro of different classes of immune effector cell by an intact bsAb. In addition to T lymphocytes that are recruited by one arm of BiUII, the Fc region binds and activates $Fc\gamma RI^+$ cells, such as monocytes/macrophages and DC. This was demonstrated by the induced production of IL-1β, IL-2, IL-6,

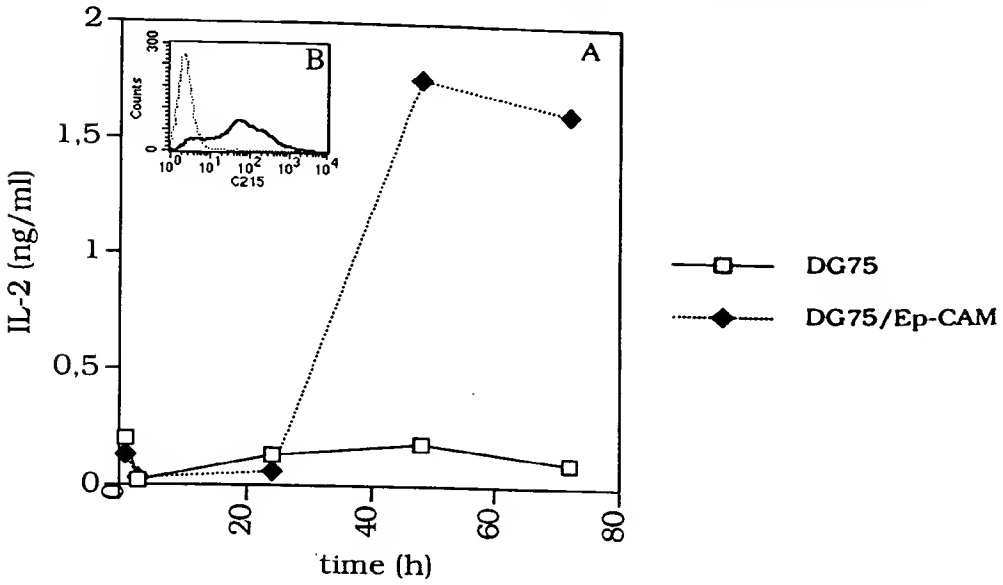

FIGURE 7. Significant levels of IL-2 are only produced in the presence of the target molecule, Ep-CAM. Allogeneic PBMCs (1×10^5) were incubated with DG75 or stably transfected DG75/Ep-CAM cells (1×10^4). IL-2 production was measured by a bioassay as indicated in *Material and Methods* (*A*). Expression of Ep-CAM was determined by FACS analysis using the monoclonal C215 Ab. Whereas Ep-CAM is not expressed on DG75 B cells, it is present on a stably transfected subclone (DG75/Ep-CAM) as revealed by FACS analysis (*B*).

IL-12, and DC-CK1 (Figs. 6 and 8). Obviously, this strong activation potential is correlated to the subclass combination mouse IgG2a × rat IgG2b that, in contrast to other reported combinations (e.g., mouse IgG2a × mouse IgG1 or rat IgG2b × mouse IgG1) (26), not only binds but also activates accessory cells.

Although T lymphocytes are believed to be the most important class of immune cell for the elimination of tumor cells, their activation depends on the presence of certain cytokines (most important IL-2) and so-called costimulatory molecules that are usually not delivered by tumor cells themselves. Rather, T cell activation depends on proper Ag presentation of tumor cell-derived peptides by professional APCs that have been demonstrated recently to be required for the induction of a long-lasting tumor immunity (27). New data corroborate the importance of costimulatory molecules for the prevention of activation-induced T cell anergy in immunotherapeutical trials (28). These are reasons that argue for our bispecific molecule, BiUII, that causes the simultaneous activation of both T cells and accessory cells.

Clinical trials with the 17-1A mAb demonstrated that CDC (complement dependent cytotoxicity) and ADCC most likely contribute to the observed antitumor effect. However, the most important class of effector cells, T lymphocytes, is not activated by this Ab (16). This may be a drawback because our in vitro assays with a comparable anti-Ep-CAM mAb revealed reduced activity in terms of tumor cell killing when compared with BiUII (Fig. 2,). These results are consistent with investigations in different mouse tumor models demonstrating the benefit of the redirection principle by bsAb (Fig. 9) as compared with parental mAbs in vivo (29–32).

We also show by different methods that BiUII is much more efficient in inducing cytokine production than the two parental Abs (Fig. 6). Although the parental anti-Ep-CAM mAb is able to induce an antitumor ADCC (data not shown), an elevated cytokine production is not observed. The same holds true for the IL-2 production by T cells, which was only slightly increased after addition of the anti-CD3 mAb 26II6 in our experimental setting. Also, the BiUII-mediated production of IL-2 depends on the presence of the target Ag Ep-CAM. Therefore, our in vitro assays demonstrate that the interaction of T cells and accessory cells alone in the absence of Ep-CAM-positive target cells is not sufficient for the induction of IL-2 (Fig. 7), a fact that reduces the risk of intolerable systemic

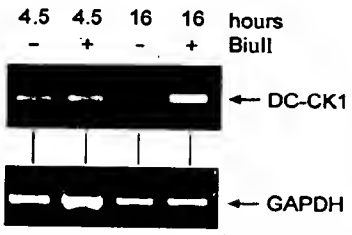

FIGURE 8. Activation of dendritic cells by BiUII. DC were incubated with PCI-1 either with or without BiUII. Whereas 4.5 h of culture seems to be insufficient for the induction of the DC-specific cytokine DC-CK1 expression, elevated expression of DC-CK1 is evident after 16 h of culture.

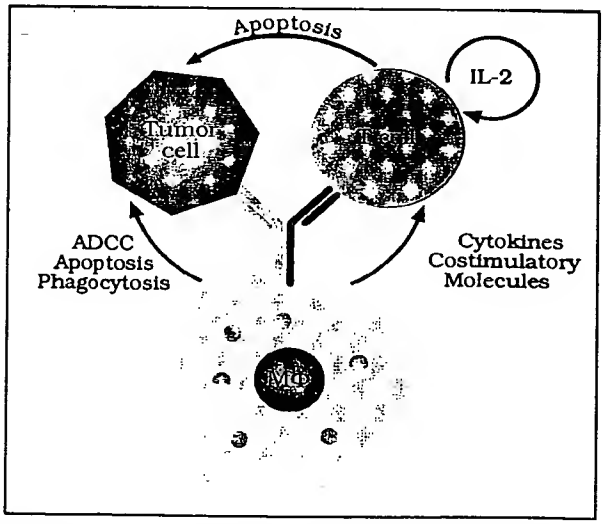

FIGURE 9. The postulated Tri-cell-complex. The addition of BiUII leads to the simultaneous recruitment of tumor cells, T cells and FcγRI$^+$ accessory cells, resulting in the activation of the latter two populations and in an efficient tumor cell killing which clearly exceeds that of the two parental Abs.

IL-2 concentrations during in vivo application of the bsAb. However, in strong contrast to other reported bsAb, BiUII activates T cells and accessory cells without the addition of exogenous IL-2, a fact that is particularly important for in vivo Ab application. In contrast, Klein et al. (8) demonstrated recently that exogeneous IL-2 is necessary for a comparable activation of immune cells in vitro with the intact bsAb, SHR-1. Because SHR-1 is composed of the subclasses mIgG1 × rat IgG2b, these data argue for the necessity of two potent subclasses (like mIgG2a × rat IgG2b) to obtain optimal effector function leading to activation of accessory cells. Thus, the Tri-cell-complex represents a self-supporting system that produces cytokines that are necessary for efficient immune cell activation.

Thus, we postulate that the BiUII-mediated activation of accessory cells via its Fc region in addition to the recruitment of T cells contributes in two different ways to tumor cell destruction: 1) The contribution of accessory cells in the absence of T cells was demonstrated by cytotoxicity assays using the adherent fraction of PBMCs (Fig. 3). These data indicate that direct lytic killing mechanisms like apoptosis and phagocytosis or generally spoken, ADCC, exerted by different classes of accessory cells, improve tumor elimination. This holds true especially at decreasing E:T ratios as shown in Fig. 4, where a $CD2^+$ subset was compared with whole PBMC. 2) The activation of accessory cells is demonstrated by the production of cytokines like DC-CK1, IL-1β, IL-6, and IL-12. Our results provide evidence that the simultaneous activation of different effector cells clearly multiplies the antitumor efficiency (Figs. 2, 5, 6, and 8). Hence, we postulate that the formation of a Tri-cell-complex consisting of T cells, accessory cells, and tumor cells is required for optimal antitumor efficiency (Fig. 9), because a single class of effector cells or a combination of the two parental Abs (Fig. 2) was much less efficient.

bsAb are either used as intact molecules or as $F(ab')_2$ fragments. Whereas the antitumor activity is significantly higher with intact bsAb, $F(ab')_2$ fragments are considered to provoke less serious side effects in vivo (33). An excessive production of cytokines is often regarded as the major drawback and limiting factor for in vivo application of intact bsAb. However, in our mouse model system, a comparable bsAb (anti-MHC class II/anti-CD3) with the same Ab isotypes (mouse IgG2a and rat IgG2b) was well tolerated even at concentrations much higher than those which are considered to have therapeutic effects in humans (31).

Another strong argument supporting the use of intact bsAb is the observed up-regulation of cytokines and activation of accessory cells, especially DC (Fig. 8), which are prerequisites for the induction of an antitumor immunity. Anti-Id network responses, as well as cellular and humoral immune responses, were observed in single cases even after conventional immunotherapeutical approaches with mAbs. The Tri-cell-complex seems to be an ideal environment for cell-cell interactions, allowing the exchange of costimulatory signals between T cells and accessory cells that amplifies immune reactions. In addition, we have demonstrated recently that an intact bsAb elevates a long-lasting tumor immunity in a syngeneic lymphoma mouse model (34). However, due to the xenogenic origin of BiUII, human anti-mouse Ab or human anti-rat Ab reactions cannot be excluded, especially after repeated applications. Therefore, the future aim will be to evaluate efficacy, side effects, and optimal application modes of intact bsAb to induce antitumor immunity in animal models and clinical trials.

Acknowledgments

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The Fc-region of a new class of intact bisp cific antibody mediates activation of acc ssory c lls and NK cells and induces direct phagocytosis of tumour cells

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Summary Bispecific antibodies (bsAb) are considered as promising tools for the elimination of disseminated tumour cells in a minimal residual disease situation. The bsAb-mediated recruitment of an immune effector cell in close vicinity of a tumour cell is thought to induce an antitumoural immune response. However, classical bispecific molecules activate only a single class of immune effector cell that may not yield optimal immune responses. We therefore constructed an intact bispecific antibody, BiUII (anti-CD3 × anti-EpCAM), that not only recognizes tumour cells and T lymphocytes with its two binding arms, but also binds and activates Fcγ-receptor positive accessory cells through its Fc-region. We have demonstrated recently that activated accessory cells contribute to the bsAb-induced antitumoural activity. We now analyse this stimulation in more detail and demonstrate here the BiUII-induced upregulation of activation markers like CD83 and CD95 on accessory cells and the induction of neopterin and biopterin synthesis. Experiments with pure cell subpopulations revealed binding of BiUII to CD64+ accessory cells and CD16+ NK cells, but not to CD32+ B lymphocytes. We provide further evidence for the importance of the Fc-region in that this bispecific molecule stimulates Fcγ-R-positive accessory cells to eliminate tumour cells in vitro by direct phagocytosis.

Keywords: phagocytosis; accessory cells; bispecific antibody; tumour

Bispecific antibodies are regarded as efficient tools for the immunological treatment of disseminated tumour cells in minimal residual disease situations. Usually, they are constructed to target tumour cells by a specific or tumour-associated antigen and to recruit one class of immune effector cell, either T cells or accessory cells like monocytes or natural killer cells. However, long-lasting immune reactions in vivo are much more complex and depend on the activation of different classes of immune effector cells, especially in the initial phase of the immune response. This is usually regarded as the major drawback of conventional bsAb that may not yield full immune responses at the tumour site. We have developed a new class of bispecific antibody, that is composed of the two potent subclasses mouse IgG2a × rat IgG2b. BiUII, a member of these new bispecific molecules, targets tumour cells via the pan-carcinoma antigen EpCAM and T-lymphocytes via CD3. But, in contrast to other bispecific molecules described to date (Fanger et al, 1990; Valerius et al, 1997; Weiner et al, 1995), it also binds and activates human Fcγ-receptor-positive accessory cells like monocytes/macrophages, NK cells, and dendritic cells (DCs) via its Fc-region. Activation of these accessory cells results in the upregulation of costimulatory molecules like CD40, CD80, and CD86 and the production of cytokines like IL-2, IL-6, and DC-CK1 (Zeidler et al, 1999).

Although T cells are considered to be the most important cells for tumour cell elimination, they depend on proper antigen presentation by professional antigen-presenting cells (APCs) or activated accessory cells and costimulatory molecules like CD40, LFA-3, CD80, and CD86 in the presence of cytokines such as IL-2 and IL-12 (Inaba and Steinman, 1984; Stüber et al, 1996). This reveals the importance of the subclass combination for induction of activation signals via the Fc-receptor of accessory cells. A similar T-cell redirecting bsAb, SHR-1 (anti-CD3 × anti-CD19), with the subclass combination mouse IgG1 × rat IgG2b was neither able to activate accessory cells via its Fc-region in a clinical study (de Gast et al, 1995) nor in in vitro assays without addition of exogenous IL-2 (Klein et al, 1997). Moreover, the antitumour efficiency of BiUII is strongly reduced when T cells alone are used as effector cells. We therefore postulate that only the activation of more than one class of immune effector cell is necessary to provide optimal antitumour efficiency. Furthermore, phagocytosis, processing, and presentation of tumour material by APCs are prerequisites for the induction of a polyclonal humoral and cellular antitumour immune response. These data are in accordance with the work of Clynes et al (1998), who recently demonstrated the importance of Fc receptors in passive and active immunity to a melanoma model.

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MATERIALS AND METHODS

Cell lines and PBMC preparation

PCI-1 (a gift from Dr T Whiteside, Pittsburgh, PA, USA) is an adherent squamous carcinoma cell-line of the head and neck (SCCHN) and is kept in DMEM with 10% FCS. The cell-line expresses EpCAM but lacks CD80 and CD86 as tested by flow

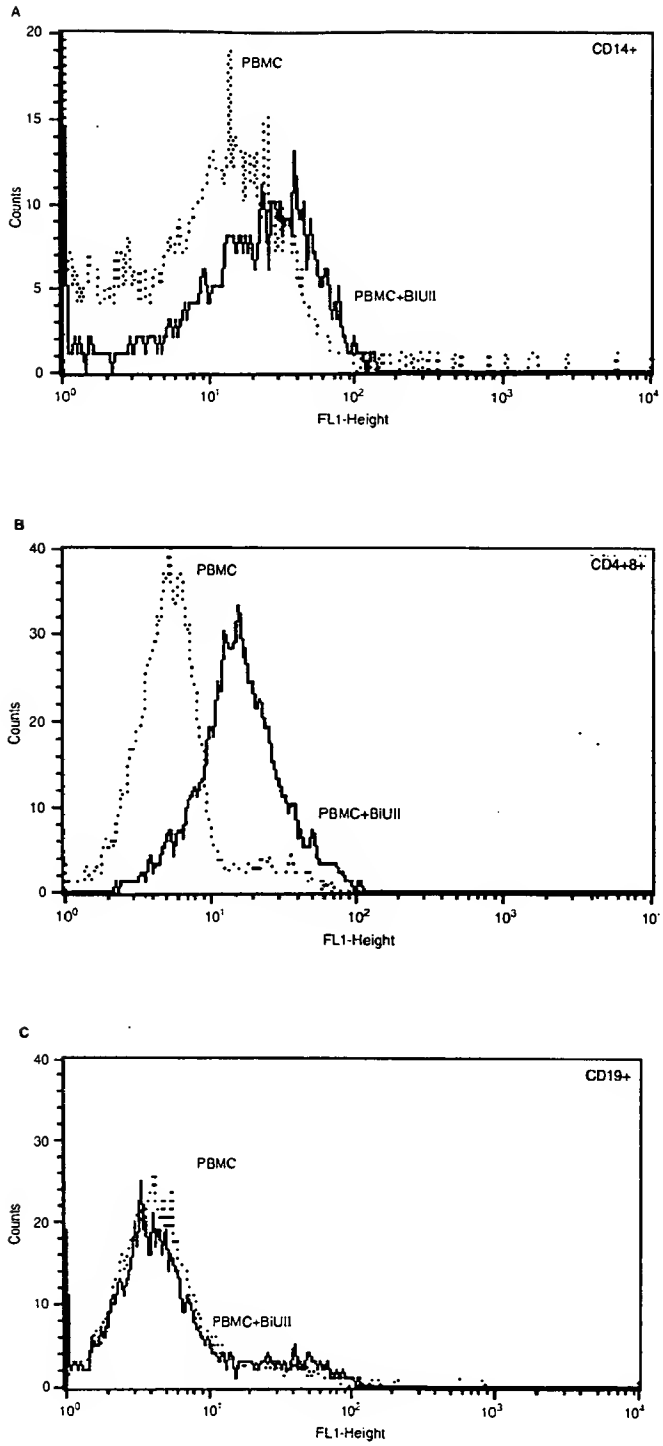

Figure 1 Binding of BiUII to PBMC subpopulations. PBMCs were incubated with BiUII and binding was assessed by FACS analysis. (A) BiUII recognizes, albeit weakly, CD14+ monocytes (black line). As monocytes do not express CD3, binding of BiUII is probably mediated by the Fc-region that binds to FcγRI with high affinity. (B) BiUII strongly binds to CD3-expressing CD4+/CD8+ T lymphocytes. (C) No binding of BiUII antibodies was observed on CD19+ B cells. Isotype control = dotted line.

cytometry (not shown). Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood of voluntary donors by Ficoll density centrifugation.

Monoclonal antibodies

mAbs for FACS analysis were from Pharmingen (Hamburg, Germany) except the DC-specific antibody BMA-X11 (Dianova, Hamburg, Germany).

Generation of dendritic cells

The adherent fraction of PBMCs was incubated for 7 days in Iscove's medium with 5% FCS (both Gibco BRL, Gaithersburg, MD, USA) and 800 U ml^{-1} each of human IL-4 and GM-CSF (both Boehringer Mannheim, Penzberg, Germany).

FACS® analysis

For FACS® analysis, 10^5 cells were incubated with the primary antibody for 30 min on ice in PBS with 2% FCS. Cells were washed twice in PBS and incubated for another 30 min with the second, FITC-labelled, antibody. After two final washings, propidiumiodide was added and flow cytometry was performed using a FACSCalibur® cytometer and the CellQuest analysis program (Becton Dickinson, Heidelberg, Germany). For isolation of highly purified $CD2^+$ cells, PBMCs were incubated with FITC-labeled antibodies and separated on a FACS-Calibur®.

Production of BiUII

The BiUII Quadroma was produced as previously described (Lindhofer et al, 1995). The following hybridomas have been used: 26II6 (rat IgG2b, anti-CD3, provided by R Schuh, GSF, Germany) and C215 (mouse IgG2a, anti-EpCAM, kindly provided by M Dohlsten, Pharmacia Upjohn, Sweden). To isolate hybrid Ab molecules of the subclass combination rat IgG2b/mouse IgG2a from quadroma, the supernatants were centrifuged, filtered, and loaded onto a 5 ml Econo Pac protein A column (Biorad, Richmond, CA, USA). After washing with 10 volumes of PBS, antibodies with the hybrid heavy-chain configuration were eluted with 0.1 M citric acid, pH 5.1.

Cell culture and killing efficiency

For determination of BiUII-mediated killing of tumour cells and cytokine production, 1×10^4 PCI-1 cells per well (targets = T) were pipetted in 96-well flat-bottom plates (Falcon) and PBMCs or subpopulations of these effectors (= E) were added at E:T ratios from 40:1 to 1:1. BiUII was used at 10 ng per well in a total volume of 100 µl per well RPMI with 10% FCS. Plates were incubated for 3 days at 37°C in a humified atmosphere and 5% CO_2.

Isolation of monocytes/macrophages and NK cells

CD14+ monocytes/macrophages and CD56+/CD3– NK cells were isolated from PBMCs using PE-labelled monoclonal antibodies and a Becton Dickinson FACSVantage cell sorter. Purity of isolated cells was examined by flow cytometry.

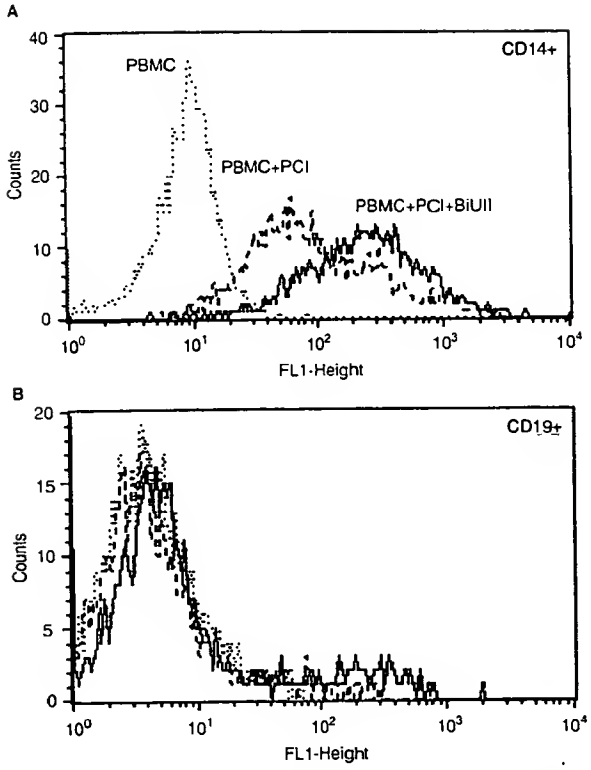

Figure 2 BiUII stimulates accessory cells to phagocytose PCI tumour cells. FITC-labelled PCI cells were cocultivated with PBMCs for 15 h with or without BiUII. After 15 h, monocytes (CD14+) and B cells (CD19+) were analysed for the presence of FITC, indicating the phagocytosis of PCI cells. (**A**) The most prominent FITC mean values (248) were observed in monocytes incubated in the presence of BiUII. A much lower intensity (76) was observed where BiUII was omitted. Background value of PBMC was 9. Similar results were observed after 24 or 48 h cocultivation period (data not shown). (**B**) In contrast, no FITC was incorporated in B cells.

FITC-labelling and uptake of PCI-1 tumour cells

PCI-1 cells were washed twice with Ca^{2+} and Mg^{2+} free PBS. I µl of FITC (1 mg ml^{-1} in Ethanol: Sigma, Deisenhofen, Germany) was then added to each 2×10^5 tumour cells in 100 µl PBS, and cells were shaken for 30 min at room temperature. Thereafter, FITC-labelled PCI-1 cells were washed twice with cell culture medium and added to PBMC cultures. The intensity of FITC-labeling was monitored by FACS analysis. Phagocytic capacity of PBMC co-incubated with FITC-labelled PCI-1 cells and BiUII was revealed by FACS after staining with PE-labelled mouse-anti-human-CD14 or –CD19 antibodies. FITC fluorescence intensity of vital CD14+ or CD19+ PBMCs was measured and interpreted as uptake of FITC-labelled PCI-1 tumour cells. Binding of BiUII to PBMC subclasses was revealed by FACS analysis after double-staining with FITC-labelled mouse-anti-rat antibodies (Dianova, Hamburg, Germany) and PE-labelled mouse-anti-human–CD4/CD8, –CD14, or –CD19 antibodies. A combination of gates (vital cells, CD14+ or CD19+ and FCS vs SSC) was used to exclude aggregates of PCI-1 cells with PBMCs from our analysis of phagocytosis of tumour cells.

MTT-Assay

To assess BiUII-mediated tumour cell killing, a colourimetric MTT-based assay was performed as previously described (Heo et al, 1990). Briefly, PCI-1 target cells were plated in wells of a 96-well flat-bottom plate and incubated overnight to prepare semiconfluent cell monolayers. Effector cells were added to the tumour cell monolayers at the appropriate ratios and plates were incubated for 24–48 h. After removing effectors by washing, MTT solution (0.5 mg ml^{-1}; Sigma) was added, and plates were incubated for a further 4 h. The MTT solution was removed and blue crystals of formazan formed in viable tumour cells were dissolved by adding dimethylsulphoxide. Plates were read at 540 nm in a spectrophotometer and results were calculated based on the mean absorbance obtained from at least six wells according to the following formula: % cell death = $100 \times (C-E)/(C-B)$, where C is the optical density reading of the cells with target cells in the absence of effectors (control), B is background without any cell population, and E is the optical density reading of adherent tumour cells remaining in the well after co-incubation with effector cells.

Activity of GTP cyclohydrolase I and cellular pterin levels

The activity of GTP cyclohydrolase I was determined in the supernatant fraction of the cell extracts (Tris/HCl, pH 8.0; 2.5 mM EDTA) after acidic iodine oxidation of the reaction product dihydroneopterin triphosphate. The neopterin phosphates were separated by ionpairing HPLC and fluorometrically detected. Cellular neopterin and biopterin were determined in aliquots of the cell extracts after acidic iodine oxidation, deproteinization by trichloroacetic acid, pre-purification by cation-exchange chromatography and separation by reverse-phase HPLC, basically as described previously (Kerler et al, 1990).

RESULTS

BiUII binds to CD3–, FcγR+ accessory cells

We constructed a new class of bispecific antibody, BiUII, that recognizes epithelial tumour cells via the pan-carcinoma antigen EpCAM (Quak et al, 1990) and redirects T lymphocytes via CD3. We have recently shown that BiUII displays an excellent antitumour activity and also that complete PBMCs are superior to a highly purified T-cell population of the same donor with regard to tumour cell killing (Zeidler et al, 1999). We therefore addressed the question whether BiUII binds peripheral blood monocytes which express the high-affinity FcγR1, CD64 and whether these accessory cells contribute to tumour cell killing. To this end, PBMCs were incubated with BiUII and a FITC-labelled anti-rat Ig antibody and binding was assessed by FACS analysis. As depicted in Figure 1, BiUII binds to CD14+, albeit weakly. Since neither antigen recognized by BiUII (CD3 and EpCAM) is present on monocytes, we concluded that binding of BiUII to FcγR-positive accessory cells is most probably mediated by the Fc region of BiUII. This finding is in agreement with data already published (Haagen et al, 1995). In parallel, we investigated the binding of BiUII to T and B lymphocytes. T lymphocytes express CD3, one

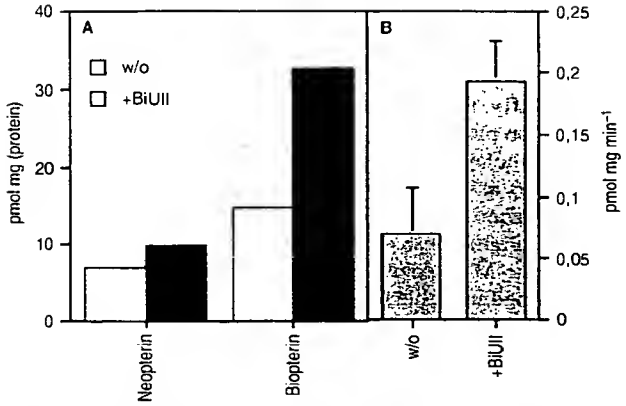

Figure 3 BiUII induces GTP-cyclohydrolase I activity and the production of neopterin and biopterin in monocytes/macrophages. PCI-1 cells were cocultivated with PBMCs either with (100 ng ml^{-1}) or without BiUII for 2 days. **(A)** The production of neopterin and biopterin and **(B)** the activity of GTP cyclohydrolase was determined. BiUII stimulates the production of both biopterin and the monocyte-specific neopterin, as well as the activity of the enzyme. PCI-1 cells per se are negative for all three products.

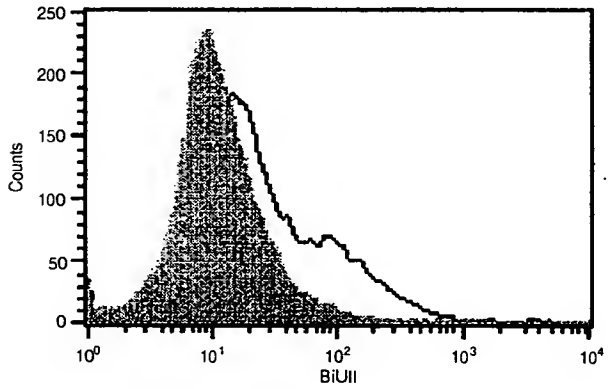

Figure 4 BiUII binds to NK cells. Binding of BiUII to highly purified, CD16+/CD3-NK cells from peripheral blood was investigated by FACS. Mean values are 64 with (black line) and 24 without BiUII (grey trace).

target molecule for BiUII, and consequently a strong binding was observed. In contrast, BiUII does not bind to CD19+ B cells that only express the low-affinity FcγRII, CD32.

BiUII-mediated phagocytosis of PCI-1 cells by CD14+ monocytes/macrophages

Since accessory cells contribute to T-cell activation and tumour-cell elimination in different ways, we wanted to find out whether direct phagocytosis of the tumour cells by CD14+ cells occurs. To this end, PCI-1 tumour cells were stained with FITC and cocultivated for 15 h with PBMCs in the presence of BiUII to assess direct phagocytosis of tumour cells. In control settings, BiUII and/or FITC-labelled tumour cells were omitted. After concultivation, the mean FITC-fluorescence intensity, indicative for the uptake of labeled PCI-1 cells, was measured in vital CD14+ monocytes or CD19+ B lymphocytes. As shown in Figure 2A, uptake of FITC-fluorescence was triggered in CD14+ monocytes/macrophages co-cultivated with BiUII. In contrast, CD19+ B cells from the same donor showed no signs for PCI-1 uptake, even in the presence of the bispecific molecule (Fig. 2B).

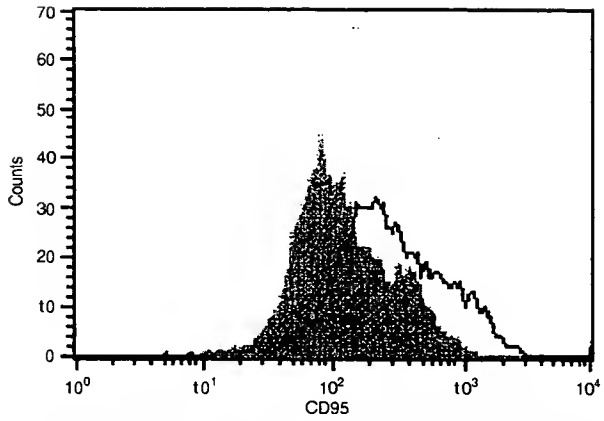

Figure 5 Addition of BiUII to a culture of purified NK cells induces the expression of CD95, an activation marker for NK cells after 1 day of culture. Mean values are 400 (black line) and 182 (grey trace) for NK cells incubated with and without BiUII, respectively.

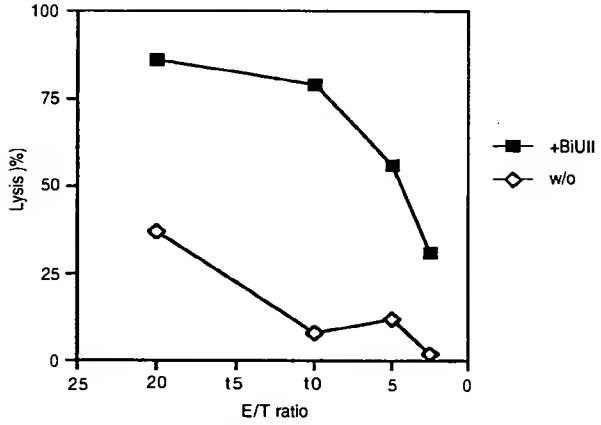

Figure 6 NK cells are stimulated to kill allogeneic tumour cells after incubation with BiUII. Highly purified CD56+CD3−NK cells (effectors) were cocultivated with PCI-1 cells at different E/T ratios for 2 days either with (100 ng ml^{-1}) or without BiUII. Killing of tumour cells (targets) was calculated in a MTT assay as described in Material and methods. One representative of three independent experiments is shown.

BiUII stimulates the production of neopterin and biopterin

Stimulation of T cells causes release of interferon-γ, which in turn induces increased expression of GTP cyclohydrolase I in monocytes/macrophages and in the T cells themselves (Schott et al, 1993; Ziegler, 1990). This enzyme initiates and controls the biopterin synthesis pathway. Therefore, activated T cells produce tetrahydrobiopterin, whereas monocytes/macrophages cannot complete the pathway. They terminate the synthesis pathway after the first step and instead accumulate and shed neopterin. Increase in the activity of GTP cyclohydrolase I and the synthesis of neopterin and biopterin are therefore indicators of monocyte/macrophage and of T-cell activation, respectively (Ziegler, 1990). Figure 3 demonstrates that BiUII induces GTP cyclohydrolase activity and enhancement of neopterin and biopterin production in PBMC after cocultivation with tumour cells in the presence of BiUII, indicative for activation of T cells and monocytes.

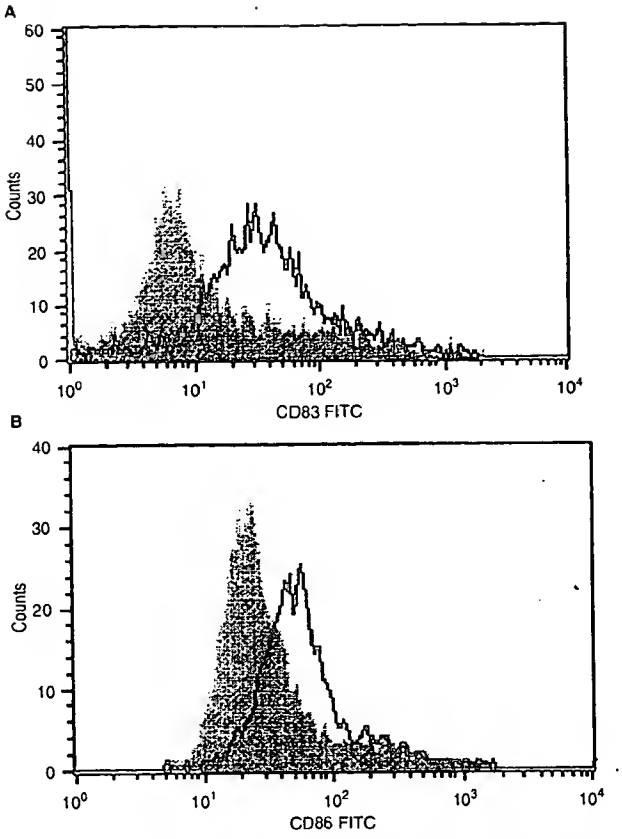

Figure 7 Induction of CD83 and CD86 on dendritic cells. Addition of BiUII for 1 day to a culture of DCs leads to upregulation of (A) the activation marker CD83 (B) and the costimulatory molecule CD86. Only cells positive for the DC marker BMA-X11 were considered for analysis. With BiUII = black line; without antibody = grey trace.

BiUII activates NK cells to tumour cell lysis

NK cells are known to play a pivotal role for the elimination of tumour cells. Since NK cells express the low-affinity FcγRIII (CD16), we wondered whether BiUII not only binds to CD64+ monocytes/macrophages but also to CD16+ NK cells. We therefore isolated highly purified CD56+/CD3– NK cells and incubated them with BiUII and revealed binding of the bispecific antibody by FACS analysis (Figure 4).

Binding of BiUII to NK cells via CD16 should lead to their activation, resulting in an antitumour activity. We therefore looked for BiUII-mediated induction of CD95 on NK cells, which is recognized as an activation marker for these cells (Medvedev et al, 1997; Robertson et al, 1995) and investigated tumour-cell killing by BiUII-activated NK cells. As shown in Figure 5, addition of BiUII to the cell culture induces the expression of CD95 on CD3–/CD16+NK cells indicating their activation via the Fc-region of BiUII. Consequently, cocultivation of NK cells with allogeneic PCI-1 tumour cells in the presence of BiUII resulted in enhanced tumour-cell killing (Figure 6). We observed that NK cells per se display a remarkable activity against allogeneic cells. However, this cytotoxicity was further enhanced by the addition of BiUII.

BiUII induces th upregulation of costimulatory molecules on dendritic cells

The network of dendritic cells (DCs) is another class of key regulators of immune responses. DCs are potent antigen-presenting cells (Steinman, 1991) and trigger the activation of T cells, e.g. via the CD40-dependent pathway (McLellan et al, 1996). Activation of DCs is characterized by the neoexpression of CD83 (Czerniecki et al, 1997; Zhou and Tedder, 1996) and upregulation of costimulatory molecules (Cella et al, 1996). Thus, DCs are thought to be involved in the generation of cytotoxic T cells (Cella et al, 1996; Ridge et al, 1998).

The objective of this study was to investigate whether DCs are activated by BiUII. DCs were generated from the adherent fraction of PBMCs by incubating these cells for 2 weeks in the presence of IL-4 and GM-CSF. The percentage of DCs in the culture was checked by staining with the DC-specific antibody BMA-X11 and was shown to be > 80% (not shown). The DCs were incubated overnight either with BiUII (100 ng ml^{-1}) or left untreated in cell culture medium only. After 16 h, the expression of surface markers CD83 and CD86 was revealed by FACS analysis. As shown in Figure 7, incubation of DCs in the presence of BiUII leads to the upregulation of both CD83 and the costimulatory signal CD86, indicating the activation of DCs mediated by our bispecific molecule.

DISCUSSION

We demonstrate here that not only the two specific binding arms but also the Fc-region of a bispecific antibody can contribute to activation of immune effector cells and thus to anti-tumour activity. However, binding of Fcγ receptors and activation of FcγR expressing cells strictly depends on the composition of the Fc-region of the bispecific molecule. Mouse IgG2a and rat IgG2b are two evolutionally related potent effector subclasses that, in combination, exert efficient activation of human accessory cells. This is shown by:

- the upregulation of costimulatory molecules and activation markers like CD83, CD86, and CD95
- the upregulation of neopterin synthesis
- the direct phagocytosis of tumour cells by purified monocytes, and
- the direct killing by isolated accessory cells without the contribution of T cells.

Interestingly, PBMCs were only weakly activated by equimolar amounts of the two parental monoclonal antibodies (Zeidler et al, 1999).

Conventional bsAbs are usually composed of one potent subclass like mouse IgG2a or rat IgG2b and a less potent subclass like mouse IgG1 (de Gast et al, 1995), or even two less potent subclasses (Weiner et al, 1993). As a consequence, the Fc-region of conventional bsAbs is usually not able to activate human accessory cells. Instead, these bispecific molecules bind and activate a single class of effector cell via one of their binding arms. This has the drawback that, in the case of T cells, an isolated activation via the CD3 molecule without appropriate costimulatory signals may cause activation-induced anergy (Daniel et al, 1998). We therefore constructed a bispecific antibody that activates more than one class of immune cell, a situation that much more resembles inflammatory and immune reactions in vivo. We have already shown the potential of such new bsAbs in tumour eradication in an animal

model (Lindhofer et al, 1996). The aim of the experiments presented here was to reveal the mechanisms that are induced by this new agent, in more detail. Therefore, we demonstrate the activation of accessory cells that either express Fcγ-RI (monocytes/macrophages and DCs) or Fcγ-RIII (NK cells). We also show that, for example, monocytes/macrophages not only are activated but also directly contribute to the anti-tumour activity of BiUII by phagocytosis. In contrast, this mouse IgG2a × rat IgG2b bispecific molecule does not bind to B lymphocytes that express the low-affinity receptor CD32. Enhanced production of tetrahydrobiopterin after BiUII stimulation may also participate in the modulation of cell functions, e.g. by increasing NO production (Mayer and Hemmens, 1997). Further, accessory cells deliver molecules like CD40, CD80, and CD86, important for T-cell activation (McLellan et al, 1996; Van Gool et al, 1996) and produce pro-inflammatory cytokines (Zeidler et al, 1999). The significance of accessory-cell activation is underlined by data recently published that demonstrates the importance of CD28 costimulation for the prevention of activation-induced T-cell death in an bsAb-immunotherapy trial (Daniel et al, 1998).

Probably most important, due to the recruitment of different immune effector cells, BiUII-mediated immune complexes represent a self-supporting system that is not dependent on the addition of exogenous IL-2, a fact that is extremely advantageous for in vivo applications. The concerted activation of T cells and accessory cells at the tumour site, leading to the phagocytosis, processing, and presentation of tumour material, may account for the potential of this new class of intact bispecific antibody and is a prerequisite for a polyclonal humoral and cellular immune response. Although HAMA or HARA reactions in vivo cannot be excluded, especially after repeated applications, this new class of bispecific molecule may represent a promising tool for the adjuvant treatment of cancer patients.

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Induction of a long-lasting antitumor immunity by a trifunctional bispecific antibody

Peter Ruf and Horst Lindhofer

Bispecific antibodies (bsAbs) can efficiently mediate tumor cell killing by redirecting preactivated or costimulated T cells to disseminated tumor cells, especially in a minimal residual disease situation. This study demonstrates that the trifunctional bsAb BiLu is able to kill tumor cells very efficiently without any additional costimulation of effector cells in vitro and in vivo. Remarkably, this bsAb also induces a long-lasting protective immunity against the targeted syngeneic mouse tumors (B16 melanoma and A20 B-cell lymphoma, respectively). A strong correlation was observed between the induction of a humoral immune response with tumor-reactive antibodies and the survival of mice. This humoral response was at least in part tumor specific as shown in the A20 model by the detection of induced anti-idiotype antibodies. Both the survival of mice and antitumor titers were significantly diminished when $F(ab')_2$ fragments of the same bsAb were applied, demonstrating the importance of the Fc region in this process. With the use of T-cell depletion, a contribution of a cellular antitumor response could be demonstrated. These results reveal the necessity of the Fc region of the bsAb with its potent immunoglobulin subclass combination mouse immunoglobulin G2a (IgG2a) and rat IgG2b. The antigen-presenting system seems to be crucial for achieving an efficient tumor cell killing and induction of long-lasting antitumor immunity. Hereby, th recruitment and activation of accessory cells by the intact bsAb is essential. (Blood. 2001; 98:2526-2534)

Introduction

Bispecific antibodies (bsAbs) are regarded as powerful tools for the treatment of malignant cells in a minimal residual disease situation, as single disseminated tumor cells are most easily targeted by an immunologic attack. However, the bsAbs described to date normally activate only a single class of effector cell, ie, either T cells,[1] natural killer cells,[2] Fcγ-receptor type I^+ cells,[3] or Fcα-receptor type I^+ cells.[4] Here, we introduce a new quality with an intact bsAb, consisting of the 2 evolutionary-related isotypes—mouse immunoglobulin G2a (IgG2a) × rat IgG2b—that are both potent in terms of immunologic effector functions. This intact bsAb is able to simultaneously redirect and activate T cells (via one arm) and accessory cells (via the Fc region) in the vicinity of tumor cells.[5] We demonstrate here that such a trifunctional bsAb can also induce effective immune responses in 2 different syngeneic mouse tumor models. Furthermore. this isotype combination leads to a high production yield of bsAbs with a simple, 1-step purification method.[6]

The successful induction of antitumor immunity was observed in various murine tumor models by using gene therapy,[7] vaccination strategies,[8,9] or antibody-mediated immunotherapy,[10,11] demonstrating the feasibility of new concepts in cancer therapy. However, the transfer of these encouraging approaches into the clinic is still hampered by certain disadvantages. One obstacle is the insufficient gene transfer in gene therapy approaches. Moreover, recent studies elucidate the complexity of T-cell regulation, revealing that, in addition to CD28, other costimulatory molecules and cytokines are necessary for appropriate T-cell activation. In this context, Renner et al[12] demonstrated the need for adhesion molecules such as LFA-1 and CD2 as costimulatory signaling molecules, rather than as pure cellular contact mediators in CD3 and CD28 bsAb-stimulated T lymphocytes. Therefore, the gene transfer of single costimulatory molecules or cytokines into tumor cells will probably not be sufficient to achieve physiologic T-cell activation at the tumor site and induction of tumor-specific memory T cells. This handicap may be overcome by fusing tumor cells with dendritic cells (DCs)[8,13] or by transferring genes encoding tumor antigens into DCs; however, both approaches are technically complex and therefore may be unsuitable for routine clinical application.

In our study, we tried to mimic the natural situation by using intact bsAbs to redirect not only T cells but also accessory cells to the tumor site, allowing a simultaneous activation of the antigen-presenting system. In contrast to other approaches, this process could be accomplished without the need for complicated gene transfer or cell fusion techniques. The trifunctional bsAb binds and activates the T cell by the CD3 molecule so that the activation signal 1 is delivered to the T cell. The activation of accessory cells is initiated by the binding to the Fc region of the bsAb by Fcγ-receptor type I[14] and the simultaneous interaction with costimulatory molecules of the T cell such as CD40L. Vice versa activated accessory cells deliver all necessary costimulatory signals to the T cell in the postulated tri-cell complex.[5] After activation by trifunctional bsAbs tumor material is phagocytosed by accessory cells as has already been demonstrated.[14] We now show the induction of a cellular and humoral antitumor immunity after application of the trifunctional bsAb, BiLu, in vivo. Thereby, the immune response

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could be directed against the tumor-specific immunoglobulin idiotype (Id) that was not targeted by the bsAb in the A20 lymphoma model. Moreover, the induced antitumor immunity was protective as demonstrated in rechallenge experiments with untransfected A20 wild-type cells that lack the human anchor protein epithelial cell adhesion molecule (EpCAM)[15] recognized by the bsAb BiLu. These data support the tri-cell complex hypothesis and make trifunctional bsAb a promising tool, especially in the treatment of malignant lymphomas.

Materials and methods

Mice and cell lines

C57BL/6 (H-2^b) and BALB/c (H-2^d) mice, 7 to 8 weeks of age, were purchased from Bomholtgaard (Ny, Denmark). The B16 cell line transfected with human EpCAM (B16-EpCAM) was kindly provided by M. Dohlsten.[16] The human EpCAM-transfected cell line 293Ep and the corresponding vector control 293Δ were kindly provided by Markus Münz (HNO Klinik Grosshadern, Munich, Germany). Stable EpCAM transfection of the A20 cell line (A20-EpCAM) was made by using the expression vector pCEP4 (Invitrogen, NV. Leek, Netherlands) deleted of the *ori P* and containing the complementary DNA of human EpCAM (kindly provided by R. Zeidler, HNO Klinik Grosshadern). B16 (CRL-6322), A20 wild-type (TIB-208), and HCT-8 (CCL-244) tumor cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). All cell cultures were maintained in RPMI 1640 media (Life Technologies, Paisley, Scotland) supplemented with 5% fetal calf serum (FCS), 50 μM β-mercaptoethanol, 2 mM glutamine, 1 × nonessential amino acids, and 100 U/mL penicillin and streptomycin (complete media). Additionally, Geneticin G418 was added to B16-EpCAM cells (0.5 mg/mL), and A20-EpCAM cells were grown in complete media containing Hygromycin B (0.5 mg/mL).

Production and purification of bsAb BiLu and bsF(ab')$_2$

The bsAb BiLu consists of the 2 parental antibodies 17A2[17] specific for murine CD3 and C215 directed to human EpCAM (kindly provided by M. Dohlsten, Pharmacia Upjohn, Uppsala, Sweden). It is an intact bsAb with the IgG subclass combination rat IgG2b × mouse IgG2a and was produced by using the quadroma technology.[18] A single-step purification with protein A was performed as described.[6] BsF(ab')$_2$ was obtained by enzymatic digestion of the purified intact bsAb with pepsin (Merck, Darmstadt, Germany). Hereby, 1 mg BiLu (0.4 mg/mL) was digested with 50 μg pepsin (10 Fip-U/mg) at 37°C and at pH 4.1 in 0.1 M acetate buffer for 7 hours. The reaction was stopped by adding 1 M Tris until pH 8 was reached. Residual intact bsAbs as well as digested Fc portions were separated from bsF(ab')$_2$ on FPLC MonoS cation exchange chromatography (Pharmacia Upjohn, Sweden). Purity and biological binding activity were confirmed by sodium dodecyl sulfate (SDS) gel electrophoresis, enzyme-linked immunosorbent assay (ELISA), flow cytometry, and cytotoxicity assays.

In vitro cytotoxicity assay

BsAb-mediated tumor cell killing was measured by a colorimetric MTT-based assay as described previously.[19] Briefly, B16 tumor cells transfected with the human EpCAM gene or wild-type B16 tumor cells were coincubated with spleen cells of naive C57BL/6 mice for 24 to 48 hours in 96-well flat-bottom plates at the indicated ratios and in the presence of 50 ng/mL bsAb. Alternatively the antibody concentration was titrated from 50 to 0.05 ng/mL at a fixed E/T ratio of 20:1. To increase T-lymphocyte frequency, spleen cells were reduced of B lymphocytes by panning with anti–IgG+M (Dianova, Hamburg, Germany). As measured by flow-activated cell sorter (FACS) the effector cell population consisted of about 50% $CD4^+$ T cells, 35% $CD8^+$ T cells, 5% macrophages, and 10% remaining B cells (not shown). Then, after removal of effector cells by washing, viable adherent B16 cells were stained with MTT solution (0.5 mg/mL; Sigma, Deisenhofen, Germany) for 4 hours. The MTT solution was removed, and blue crystals of formazan were dissolved in dimethyl sulfoxide. Absorbance was measured with a spectrophotometer at 540 nm. Results were calculated as follows: the percentage of cell death = 100 × (C − E)/(C − B), where C is the optical density reading of target cells without effectors (control), B is the background without any cell population, and E is the optical density reading of adherent tumor cells remaining in the wells after coincubation with effector cells. In all cases at least triplicates were performed, and SD was less than 15%.

FACS analysis

Target cells (2-4 × 10^5) were incubated with the primary antibody for 30 minutes on ice in FACS buffer (phosphate-buffered saline with 5% FCS and 0.1% NaN_3). After washing, cells were stained with a second fluorescein isothiocyanate (FITC)– or phycoerythrin-labeled antibody, washed, and suspended in FACS buffer with propidium iodide. Flow cytometry was performed by using a FACSCalibur cytometer and the CellQuest analysis program (Becton Dickinson, Heidelberg, Germany). For detection of bsAbs binding on murine CD3, EL-4 lymphoma or spleen cells from mice were used as targets. Binding to human EpCAM was assessed with the transfected cell lines A20-EpCAM, B16-EpCAM, 293Ep, or with human HCT-8 cells. Polyclonal antimouse IgG, antirat IgG (Dianova, Hamburg, Germany), or monoclonal anti–rat IgG2b (ATCC/Tib174) and antimouse IgG2a (R19-15, Pharmingen, Hamburg, Germany) were used as secondary detection antibodies.

Assessment for tumor-reactive antibodies

The presence of tumor-reactive antibodies in mice sera was determined by flow cytometry. For this purpose mice sera were diluted 1:30 in FACS buffer and incubated with 2 to 4 × 10^5 B16 or A20 target cells. After washing antibodies bound to tumor cells were detected either by FITC-conjugated polyclonal rat anti–mouse IgG or with IgG subclass-specific antibodies against mouse IgG1 and IgG2a (Pharmingen, Hamburg, Germany). Reactivity was calculated as the percentage of positively stained tumor cells. The nonparametric Mann-Whitney *U* test was used to evaluate statistical differences between serologic reactions.

Detection of EpCAM-specific antibodies

Preimmune and postimmune sera of mice were pooled in groups and titrated against human EpCAM-transfected 293Ep cells. Cell-bound antibodies were detected by flow cytometry using a FITC-conjugated goat antimouse IgG1 antibody (Southern Biotechnology Associates, Birmingham, AL). EpCAM specificity of detected antibodies was verified by serum titration against the 293Δ cell line that does not express the EpCAM antigen (vector control).

Anti-idiotypic ELISA

Antibodies against the A20 Id were measured as follows: ELISA plates were coated with A20 IgG2a purified from culture supernatants, incubated with serially diluted preimmune or immune sera, followed by biotin-labeled goat antimouse IgG1 (Amersham Life Science, Buckinghamshire, United Kingdom), and developed with avidin-peroxidase. Reactivity of the sera with the constant domains of the A20 immunoglobulin was excluded by a similar ELISA using the irrelevant BALB/c-derived IgG2a monoclonal antibody TPA02 (TRION Pharma GmbH, Munich, Germany) as the capturing antigen. Quantification of the anti-Id levels was performed by using the 6D4 (H.L., unpublished data, July 1993) mIgG1 anti-Id antibody against the mIgG2a 7D6[20] antibody as a standard.

In vivo therapy with bsAb

C57BL/6 mice received 5 × 10^3 B16-EpCAM melanoma cells intraperitoneally on day 0. BsAb treatment was started with 2.5 μg BiLu on day 2 and continued with 1 μg each on days 4 and 7. Alternatively, 10 μg bsAb was given on day 2 and another 5 μg on days 4 and 7. The parental group was treated with a combination of the 2 monospecific antibodies C215 and 17A2 by using equivalent doses for each antibody. Control groups received no

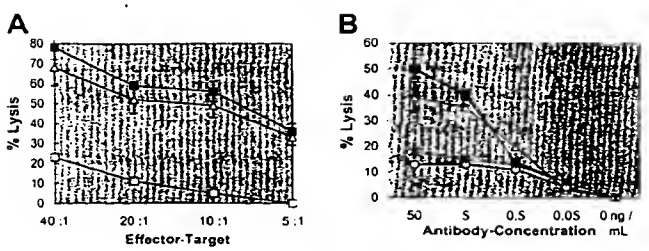

Figure 1. BsAb-mediated cytotoxicity in vitro. Tumor cell killing was measured by a colorimetric MTT-based assay. (A) Varying E/T ratios were carried out with a constant amount of 50 ng/mL bsAb BiLu (■) or bsF(ab')$_2$ (△) targeted against transfected B16-EpCAM cells. To differentiate antigen-independent cell lysis by the bsAb, nontransfected B16 target cells were used (bsAb wild-type, □). (B) At an E/T ratio of 20:1, the bsAb BiLu (■) and bsF(ab')$_2$ (△) were titrated from 50 to 0.05 ng/mL. Controls with the parental antibodies were performed at equimolar amounts (17A2+C215, ✳; and C215, ○). B16-EpCAM cells were used as targets. Effector cell-induced background lysis in the absence of antibody was subtracted.

antibody. Surviving mice were rechallenged with a reduced but still lethal amount of 750 B16-EpCAM cells. Mice were killed after apparent intraperitoneal tumor growth (abdominal swelling) that was confirmed by postmortem dissection. BALB/c mice were challenged with 2×10^6 A20-EpCAM cells intravenously followed by intraperitoneal injection of 4 μg bsAb BiLu or bsF(ab')$_2$ 3 hours later. Again the control with parental antibodies was performed. In all cases at least 2 independent experiments were carried out with a minimum of 6 mice per group. Statistical analysis of survival data was performed by using the log-rank test.

Vaccinations and adoptive serum transfer

Female BALB/c mice 10 to 12 weeks old were immunized against the syngeneic A20 tumor line by intraperitoneal injection of 5×10^4 irradiated (50 Gy) A20-EpCAM or A20 wild-type cells 2 hours after intraperitoneal donation of 4 μg bsAb BiLu or bsF(ab')$_2$ (priming immunization). Four weeks later mice received an identical booster immunization. To evaluate the role of CD4$^+$ T-helper (T_H) cells in the immunization process a group of mice was depleted of CD4$^+$ T cells by injection of 400 to 750 μg CD4.2 antibody[21] every 3 days before priming and booster immunization. CD4$^+$ or CD8$^+$ T-cell depletion in the effector phase was performed with 500 μg CD4.2 or CD8.2 antibody[21] 4 days before the tumor challenge. For control, mice were treated only with irradiated tumor cells without bsAb. The final challenge consisted of 7×10^5 viable, untransfected wild-type A20 cells and was performed (intraperitoneally) 6 weeks after the priming immunization. Alternatively, 4×10^5 A20 cells were given intravenously. Blood samples were collected from the tail vein before any treatment (control) and 2 days before the challenge. For adoptive transfer experiments sera of bsAb-immunized mice were pooled, and 300 μL serum was injected intravenously together with 3×10^5 A20 wild-type cells into naive BALB/c mice. Control mice received serum of naive nonimmunized animals in combination with tumor cells. Each experiment has been repeated at least once. All animal groups comprised 6 mice. Statistical analysis of survival curves was performed by using the log-rank test.

Results

Intact bsAb BiLu reveals high antitumor efficacy in vitro and in vivo

To evaluate the efficacy of the intact bsAb. BiLu, with specificities anti-CD3 × antihuman EpCAM in redirected lysis of tumor cells, cytotoxicity experiments were carried out in vitro. Spleen cells of naive C57BL/6 mice with an increased T-cell frequency of about 85% were obtained by removing B cells by anti–IgG+M panning. Different than described by others,[22,23] thus obtained T cells were not preactivated by interleukin 2 (IL-2) or other stimulatory molecules. Nevertheless, the melanoma line B16-EpCAM was efficiently killed by these effector cells in the presence of bsAb BiLu (Figure 1A). Furthermore, bsAb-mediated lysis was much greater than that achieved by using the parental antibodies at equimolar amounts and was observed over a wide range of bsAb concentrations. Activity was still detected at 5 ng/mL (Figure 1B). Also bsF(ab')$_2$ fragments efficiently induced tumor cell killing, indicating comparable biological activity (Figure 1A,B). Finally, the cell-mediated lysis was mainly antigen specific because B16 wild-type cells that do not express the target antigen EpCAM were only weakly killed (Figure 1A). However, there was a tumor growth inhibition of B16 cells especially at higher E/T ratios. Although we do not know the exact mechanism of this inhibition, bystander effects such as the release of cytokines like tumor necrosis factor α or interferon γ might be responsible for this observation.[24] In summary, the bsAb BiLu revealed high and predominant antigen-specific lytic capacity for syngeneic tumor cells in vitro.

As a next step, we analyzed the antitumor efficiency of the bsAb BiLu in vivo. In a therapeutic approach we injected C57BL/6 mice with a lethal dose of B16-EpCAM melanoma cells intraperitoneally and initiated bsAb therapy 2 days later. A total dose of 4.5 μg (2.5/1/1 [2.5 μg on day 2, 1 μg on day 4, and 1 μg on day 7 after tumor challenge]) was sufficient to cure 100% of the animals, whereas all control mice died within 28 days (Figure 2A). In addition, the therapeutic outcome of the parental group, which received 4.5 μg of each parental antibody, was significantly worse ($P < .006$) as compared with the bsAb group. In contrast to the observed tumor growth inhibition of untransfected B16 cells in vitro such an effect was not seen in vivo: the growth of untransfected B16 wild-type tumor could not be inhibited or delayed by the bsAb (Figure 2A). This finding clearly demonstrates the high specificity of bsAb BiLu-induced tumor cell killing and underlines the importance of retargeted cytotoxicity. To further evaluate whether these results would be similar with other tumors, we repeated this experiment in a B-cell lymphoma model with EpCAM-transfected A20 cells. A single dose of 4 μg bsAb BiLu was sufficient to inhibit tumor growth with 100% survivors, whereas an equimolar amount of both parental antibodies led to a significantly worse inhibition of tumor growth, with 29% survivors ($P = .0009$; Figure 2B). These results clearly demonstrated the benefit of the redirection principle by this bsAb in the B16 melanoma and A20 lymphoma models. Hence, our data are in good accordance with the findings of other groups that observed the same superiority of bsAb compared with parental, monospecific antibodies in different lymphoma models.[25-28]

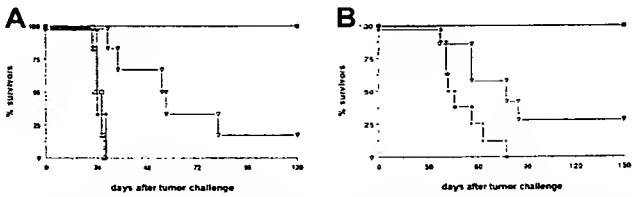

Figure 2. The bsAb BiLu reveals high antitumor activity in vivo in 2 different syngeneic tumor models. (A) C57BL/6 mice (n = 6) received 5×10^3 B16-EpCAM cells intraperitoneally on day 0. BsAb treatment was started with 2.5 μg on day 2 and continued with 1 μg each on days 4 and 7 (■). The group that received parental antibodies (▽) (n = 6) was treated with a combination of the 2 monospecific antibodies C215 (antihuman EpCAM) and 17A2 (antimurine CD3). The control group (+) (n = 6) received no antibody. To clarify the antigen dependency of the treatment one group of mice (□) (n = 6) was challenged with 5×10^3 untransfected B16 cells and injected with bsAb as indicated above. Experiments were repeated 3 times with similar results. (B) BALB/c mice were challenged with 2×10^6 A20-EpCAM cells intravenously followed by intraperitoneal injection of 4 μg bsAb (■) (n = 12) or bsF(ab')$_2$ (◆) (n = 8) 3 hours later. Again, a control group with parental antibodies (▽) (n = 7) was included, and data were confirmed by another independent experiment.

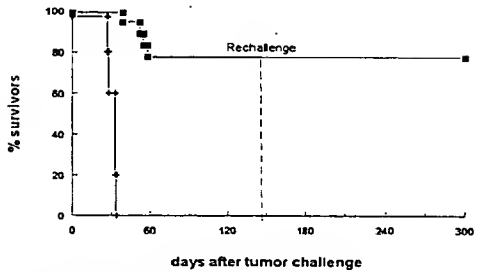

Figure 3. BsAb-treated mice develop long-lasting entitumor protection. The survival of mice is shown after donation of 5 × 10^3 B16-EpCAM cells intraperitoneally on day 0 followed by the application of 10 μg bsAb BiLu (■) on day 2 and another 5 μg on days 4 and 7. The control group received no antibody (+). Mice surviving the first tumor challenge (14 of 18 mice) were rechallenged on day 144 with a minimal lethal dose of 750 B16-EpCAM cells intraperitoneally. This time no bsAb treatment was performed. Whereas all control mice (n = 5) developed e tumor (not shown), mice pretreated with the bsAb BiLu successfully rejected the second tumor challenge. The experiment was repeated twice with similar results.

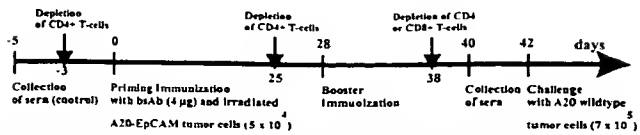

Figure 4. Immunization scheme of BALB/c mice. Mice were immunized with 4 μg bsAb BiLu followed by 5 × 10^4 irradiated A20-EpCAM cells given intraperitoneally 2 hours later. Then, after 4 weeks an identical booster immunization was performed, end finally mice were intraperitoneally challenged another 2 weeks later with 7 × 10^5 A20 wild-type cells. The role of the T cells was investigated by antibody-induced depletion of $CD4^+$ or $CD8^+$ T cells with 400 to 750 μg CD4.2 antibody and 500 μg CD8.2 antibody, respectively, at indicated time points. From all mice blood samples were collected before any treatment (control sera) as well as 2 deys before tumor challenge.

BsAb-treated mice develop tumor-reactive antibodies and long-lasting antitumor protection

In a second therapy experiment, 14 of 18 bsAb-treated mice survived the primary B16-EpCAM tumor challenge (Figure 3). To analyze differences in immune responses between mice that succumbed to the tumor and those that successfully rejected it, we assessed the sera for tumor-reactive antibodies. Indeed, we found a strong humoral response specific for the tumor in all surviving animals. In contrast, sera of mice that did not survive displayed only a weak reaction (Table 1, mice 15-18). Analysis of the immunoglobulin subclass composition revealed a dominant IgG2a response, whereas no IgG1 antitumor antibodies could be detected. To determine whether antitumor protection was also generated in the surviving animals, we challenged the mice a second time with a lethal number of tumor cells in the absence of bsAb. All animals survived the tumor rechallenge (Figure 3). Consequently, the initial treatment of the tumor with this bsAb led not only to total tumor eradication but also to the induction of immune protection. Notably, all the mice rechallenged on day 144 after the primary challenge were still able to reject the tumor, indicating the high efficacy and long duration of the antitumor response.

BsAb BiLu induces antitumor immunity in the A20 lymphoma model

Next, we investigated the exact contribution of humoral and cellular immune responses to the observed antitumor immunization. Having established that the combination of BiLu and vital tumor cells induce an antitumor response, it was important to adapt the immunization protocol to the clinical situation of tumor patients. Therefore, we designed a vaccination strategy with a defined dose of irradiated, proliferation-incompetent tumor cells. We also switched from the aggressively growing B16 melanoma model to the more moderate proliferating A20 lymphoma model. In these experiments BALB/c mice were immunized with irradiated A20-EpCAM cells and bsAb BiLu. Finally, we challenged the mice deliberately with untransfected A20 wild-type cells to see whether an immune response against the whole tumor cell independent of the target antigen EpCAM could be achieved (Figure 4). Again, prior to challenge tumor-reactive antibodies were detectable in the

Table 1. Detection of tumor-reactive antibodies in the B16 melanome model

Mice	% reactivity with B16-EpCAM cells (sera on day 0)	% reactivity with B16-EpCAM cells (sera immediately before death)	% reactivity with B16-EpCAM cells (sera on day 143)*	mIgG2a†	mIgG1†
1	5		87	+	–
2	6		42	+	–
3	5		70	+	–
4	4		73	+	–
5	5		30	+	–
6	5		66	+	–
7	5		57	+	–
8	6		71	+	–
9	5		47	+	–
10	5		49	+	–
11	6		69	+	–
12	5		40	+	–
13	5		54	+	–
14	ND		ND	ND	ND
15	4	8		–	–
16	5	14		–	–
17	6	10		–	–
18	5	18		+	–
Control serum	3	4	2	–	–

EpCAM indicates epithelial cell adhesion molecule; IgG, immunoglobulin G; ND, not done.

*Measurements were performed by flow cytometry. Tumor-reactive antibodies were detected with polyclonal fluorescein isothiocyanate-conjugated rat antimouse IgG antibody. Numbers represent percentage of positively stained cells.

†Measurements of tumor-reactive sera were done as described in above footnote, but detection followed with IgG subclass-specific antibodies against mouse IgG2a and mouse IgG1.

Table 2. Detection of tumor-reactive antibodies in the A20 lymphoma model

Group	Mice	% reactivity with A20 cells (preimmune sera)	% reactivity with A20 cells (sera after vaccination on day 40)	Vaccination treatment
A	1	1	40	Application of intact bsAb BiLu and irradiated tumor cells
	2	1	46	
	3	1	18	
	4	1	29	
	5	2	28	
	6	1	30	
B	7	2	4	Application of intact bsAb BiLu and irradiated tumor cells; depletion of $CD4^+$ T cells
	8	1	14	
	9	2	9	
	10	2	5	
	11	2	3	
	12	2	15	
C	13	1	1	Application of $bsF(ab')_2$ fragments and irradiated tumor cells
	14	2	1	
	15	2	1	
	16	2	1	
	17	2	2	
	18	1	2	
D	19	1	2	Control: application of irradiated tumor cells without antibody
	20	1	3	
	21	2	4	
	22	1	1	
	23	1	1	
	24	1	1	

bsAb indicates bispecific antibody.

Sera of immunized BALB/c mice were assessed for tumor-reactive antibodies by flow-activated cell sorter analysis as described in "Materials and methods." The reaction is shown as the percentage of positively stained tumor cells. $P < .0023$ for the differences between group A and the other groups B-D, by the Mann-Whitney U test.

sera of BiLu-treated mice but not in control mice treated only with irradiated tumor cells (Table 2). These data indicated that the intact bsAb was essential for the generation of the humoral antitumor response. Moreover, the presence of induced antibodies against the tumor correlated with the survival of mice. All control mice died, whereas animals treated with intact bsAb and developing tumor-reactive antibodies survived the tumor challenge (Figure 5).

We further analyzed the mice sera for EpCAM-specific antibodies. Interestingly, the human antigen was not immunogenic per se. Only after injection of intact bsAb BiLu and A20-EpCAM cells, antibodies against human EpCAM were generated (Table 3). Thereby, the induction of an idiotypic network response can be excluded, because the donation of BiLu in combination with $EpCAM^-$ A20 wild-type cells did not lead to an immune response against the tumor antigen. Remarkably, mice developed a humoral response and were protected against wild-type A20 cells, although they were immunized with transfected A20-EpCAM cells. As a consequence, an antitumor response against antigens other than the target antigen EpCAM must have been induced. An absolutely tumor-specific antigen of B-cell lymphomas is the immunoglobulin Id. To investigate whether this antigen was targeted, we also looked for anti-Id antibodies. Indeed, we found significant titers against the A20 immunoglobulin Id after we immunized mice with bsAb BiLu and A20-EpCAM cells according to our vaccination protocol (Figure 6). However, anti-Id antibodies were not detectable in the sera of control mice that had been immunized solely with irradiated A20-EpCAM cells in the absence of bsAb. However, the application of BiLu and A20 wild-type cells resulted in a weak anti-A20 Id response, too. Challenging these mice with A20 wild-type cells revealed a survival rate of 50% (Figure 7). This result suggests that

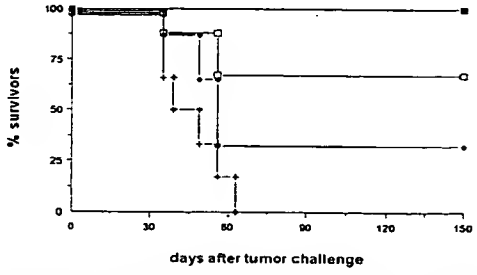

Figure 5. BALB/c mice immunized with intact bsAb BiLu and irradiated A20-EpCAM cells successfully reject A20 wild-type challenge. Mice were immunized as indicated in Figure 4 with bsAb (■) or $bsF(ab')_2$ fragments (◆) and irradiated A20-EpCAM cells. The control group was treated only with irradiated tumor cells without bsAb (+). The CD4 depletion group (□) was identically treated to the bsAb group but injected with $CD4^+$ T-cell–depleting CD4.2 antibody. In 2 independent experiments similar data were obtained.

Table 3. Antihuman epithelial cell adhesion molecule response in treated mice

Serum pool of immunized mice*	Reactivity	
	293Ep cells	293Δ cells (vector control)
A20-EpCAM	0†	0
A20-EpCAM + BiLu	240	0
A20-EpCAM + $bsF(ab')_2$	0	0
A20WT + BiLu	0	0

*Mice were immunized according to Figure 4. Sera of mice were pooled in groups and titrated against human epithelial cell adhesion molecule (EpCAM), expressing 293Ep cells and nonexpressing 293Δ cells (vector control), respectively. Cell-bound antibodies were detected by flow cytometry using a fluorescein isothiocyanate-conjugated goat antimouse immunoglobulin G1 antibody.

†Data reflect serum dilutions at which percentage of positively stained cells was still 3 times above background reaction with control sera (serum pool before immunization).

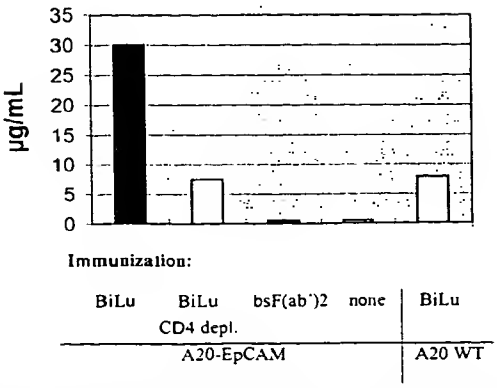

Figure 6. Antibodies directed against the A20 immunoglobulin Id could be detected in sera of BALB/c mice immunized with intact bsAb BiLu and A20-EpCAM cells. This response was clearly diminished after depletion of CD4+ T cells in the immunization phase. The use of A20 wild-type cells and intact bsAb resulted in a weak reaction, too. For the anti-idiotypic ELISA pooled and serially diluted preimmune or immune sera were incubated on A20 Id-coated ELISA plates, followed by biotin-labeled goat antimouse IgG1 and developed with avidin-peroxidase. Preimmune sera revealed no reaction.

target-antigen–independent effects are also part of the bsAb-mediated antitumor immunization, but full tumor protection (100% survival) is only accomplished after specific immunization with bsAb-targeted A20-EpCAM cells (Figure 7). In summary, the injection of the trifunctional bsAb BiLu raised humoral responses against at least 2 different tumor-specific antigens, A20 Id and EpCAM.

To determine whether this humoral antitumor response contributed to the observed protection against the A20 wild-type challenge, we performed adoptive transfer experiments. Sera of bsAb-immunized mice were pooled and transferred together with vital A20 cells into naive BALB/c mice. Although the protection effect was moderate, tumor growth in these mice was delayed significantly when compared with control mice that received A20 cells in sera of unimmunized animals ($P = .018$). This demonstrated that the obtained antitumor protection was mediated at least in part by the humoral reaction against the tumor (Figure 8).

Because the production of antibodies against tumor-specific or -associated antigens by B lymphocytes requires the support of $CD4^+$ T_H cells, we expected to suppress this reaction by the application of a depleting anti-CD4 antibody during the immunization phase. In fact, the generation of antibodies against A20 cells, in general, as well as specific anti-idiotypic antibodies was diminished after depletion of T_H cells (Table 2, Figure 6), resulting also in a significantly reduced number of surviving animals (Figure 5 and Figure 9; $P = .02$). Therefore, T_H cells were mandatory for bsAb-based induction of tumor-reactive antibodies as well as for tumor protection. Next, we evaluated the role of T cells in the effector phase. To this end, the depletion of $CD8^+$ T cells caused a decrease of survival rate from 100% to 66% (Figure 9), whereas the depletion of $CD4^+$ T cells had no effect (not shown). This finding indicated the participation of cytotoxic $CD8^+$ T lymphocytes in the eradication of the tumor. Taken together, these results demonstrated the generation of humoral as well as cellular immunity against the A20 lymphoma induced in the presence of intact bsAb BiLu.

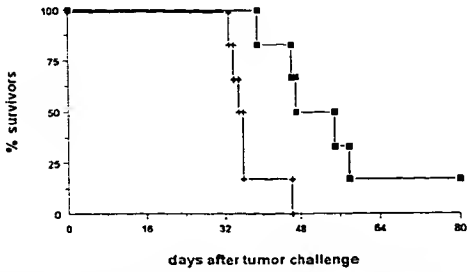

Figure 8. Adoptive transfer of immune serum into naive BALB/c mice significantly delays tumor growth ($P = .018$). Sera taken from mice 6 weeks after immunization with bsAb BiLu (■) and irradiated tumor cells were pooled and transferred (300 µL) together with 3×10^5 viable A20 cells intravenously into naive BALB/c mice. The control group (+) received serum of naive, untreated mice in combination with tumor cells.

Fc region of the bsAb BiLu is obligatory for the induction of anti-A20 immunity and efficient tumor cell killing

To clarify the role of the Fc region of the bsAb for the induction of antitumor immunity, we evaluated the efficacy of $F(ab')_2$ fragments of BiLu. Complete bsAb was digested with pepsin under limiting conditions and purified on FPLC ion exchange chromatography. FACS analysis and SDS gel electrophoresis proved that biologically active $bsF(ab')_2$ fragments of high purity were obtained (not shown). This finding was confirmed by in vitro cytotoxicity assays in which the mediated tumor cell killing proved to be comparable to intact bsAb (Figure 1). However, these $bsF(ab')_2$ fragments failed to induce an immune protection against A20 lymphoma cells in vivo. Neither tumor-reactive, EpCAM-specific, nor anti-A20 Id antibodies were detectable nor was fast performance liquid chromatography effective protection against the tumor challenge observed

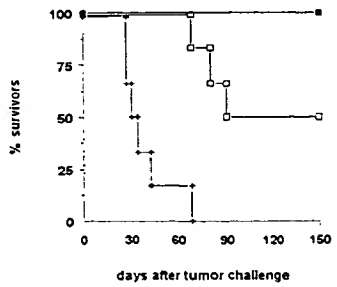

Figure 7. Specific versus nonspecific immunization. To differentiate between target-antigen–dependent and –independent immunization effects induced by the bsAb BiLu, mice were immunized as outlined in Figure 4 with bsAb and irradiated A20-EpCAM cells (■), or with bsAb and nontransfected A20 wild-type cells (□). Only the use of A20-EpCAM cells resulted in full tumor protection. Control mice (+) that received irradiated A20 wild-type cells without bsAb revealed no protection. Each group comprised 6 mice.

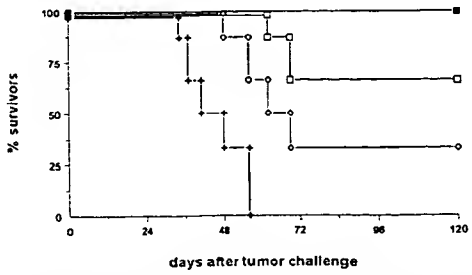

Figure 9. The depletion of $CD8^+$ T cells in the effector phase reduces tumor protection. Moreover, depletion of $CD4^+$ T cells in the priming phase results in a significant loss of tumor rejection ($P = .02$). BALB/c mice (n = 6) were vaccinated according to the immunization protocol outlined in Figure 4. Depletion of T cells was performed with injections of CD8.2 antibody during the effector phase (□, CD8-E) or with CD4.2 antibody during the immunization phase (◇, CD4-I). Control mice (+) were not pretreated. All mice received an intravenous tumor challenge of 4×10^5 A20 cells. ■ indicates bsAb.

(Tables 2 and 3, Figures 5 and 6). These data underline the essential role of the Fc part for the development of protective immunity.

Weiner et al[29] compared genetically constructed bsF(ab')$_2$ with intact bsAb redirecting CD3 T cells to the 38C13 lymphoma Id. They reported increased T-cell activation and significantly improved therapeutic outcome when complete bsAb was used. In this context, we were also interested in evaluating the direct tumor killing capacity of bsF(ab')$_2$ compared with the intact bsAb BiLu in a therapeutic setting. Therefore, we challenged BALB/c mice with A20-EpCAM cells and began with antibody treatment (4 μg) 3 hours later. Whereas the application of bsAb BiLu resulted in a 100% protection against the tumor, all mice in the bsF(ab')$_2$ group succumbed to the lymphoma (Figure 2B: $P < .0001$). Even the control group receiving the mixture of parental antibodies had a better outcome in long-term survival. In summary, the Fc portion of the bsAb was of indispensable importance for the therapeutic efficacy as well as for the immunization potency in the A20 tumor model.

Discussion

In this study, we have used newly designed intact bsAbs[6] that redirect not only T cells but also Fc receptor$^+$ cells to the tumor site. On the basis of previous investigations,[5] we argue that 2 mechanisms are mainly responsible for the high antitumor efficacy observed with this reagent. First, in addition to the recruited T cells, accessory cells are activated by an interaction between the Fc region of the intact bsAb and Fcγ receptors.[14] The combination of the 2 potent isotypes, mouse IgG2a and rat IgG2b, seems to be crucial in this process. This activation of accessory cells leads to the secretion of cytokines such as IL-12, tumor necrosis factor α, and the DC-specific cytokine DC-CK1, as well as to the presentation of costimulatory molecules to the T cell.[5] Thus, T cells are postulated to be activated via signal 1 by the anti-CD3 binding arm, and all necessary costimulatory signals can be delivered by the activated accessory cells (Figure 10). Secondly, the accessory cells contribute to the tumor cell killing by using different mechanisms, including phagocytosis.[14] This concentrated attack of different immune cells leads to a significantly improved tumor cell elimination compared with the mixture of both parental antibodies in vitro (Figure 1B) and in vivo (Figure 2A,B). This holds true for the A20 B-cell lymphoma as well as for the solid-growing B16 melanoma. The essential role of the Fc region in this context could be demonstrated by the use of F(ab')$_2$ fragments of the same bsAb. These bsF(ab')$_2$ fragments had comparable lytic capacity in vitro, proving their general biological activity, but were rather ineffective in vivo. One reason for this observation may be a nonspecific activation of immune cells caused by mechanical stress during spleen cell preparation, making an Fc-mediated activation of effector cells unnecessary. Otherwise, such effects do not exist in vivo. Another reason for the inefficiency of bsF(ab')$_2$ fragments in vivo might be their shorter half-life. But even the application of increased and repeated doses to compensate for this handicap did not lead to an effectiveness as observed with intact bsAb.[29] Remarkably, after the addition of costimulatory agents such as IL-2 or staphylococcal enterotoxin B (SEB) superantigen, Weiner et al[29] were able to reach an improved killing efficacy with bsF(ab')$_2$ fragments. Recently, similar observations were described with the use of bispecific single-chain variable fragments.[30] These results support the view that the presence of costimulatory signals is

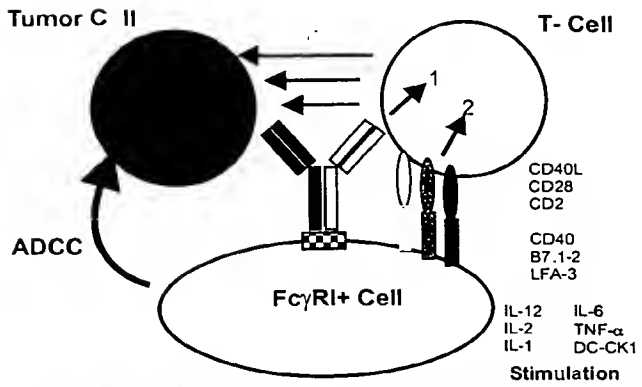

Figure 10. The postulated tri-cell complex model suggesting interactions that led to the induction of tumor immunity and improved tumor cell destruction by intact bsAb. The use of mouse IgG2a × rat IgG2b intact bsAb leads to the simultaneous recruitment of tumor cells, T cells, and Fcγ-receptor$^+$ accessory cells. The formation of this complex induces the activation of different classes of effector cells, resulting in excellent antitumor activity. The stimulation of accessory cells is demonstrated by the production of cytokines such as IL-1, IL-6, IL-12, and the DC-specific cytokine DC-CK1.[5] Activated accessory cells, particularly professional antigen-presenting cells such as DCs or activated macrophages mediate costimulatory signals, eg, via CD40-CD40L to T cells that are necessary to prevent T-cell anergy. Furthermore, tumor material is phagocytosed,[14] processed, and presented by professional antigen-presenting cells after activation by bsAb—an important prerequisite for the induction of antitumor immunity. The tri-cell complex is only a model and should not implicate a 1:1:1 ratio of the 3 involved cell types.

mandatory for full T-cell activation and efficient tumor cell killing and may explain why bsF(ab')$_2$ fragments alone are less therapeutic.

Obviously, the most interesting feature of this new intact bsAb format is its ability to induce a long-lasting tumor immunity as demonstrated by using 2 different syngeneic mouse tumor models. In the B16 melanoma model even 144 days after the first tumor contact and treatment with bsAb, a second challenge, without bsAb, was rejected in all mice, thereby demonstrating the long-lasting protection mechanism (Figure 3). In contrast, the combinatorial usage of 2 bsF(ab')$_2$ fragments (CD3 × EpCAM + CD28 × EpCAM) in the identical tumor model only led to a marginal tumor elimination and poor induction of tumor immunity.[31] This result further supports the hypothesis that the Fc portion of the bsAb is crucial for the killing capacity as well as for the induction of tumor immunity and suggests that single costimulatory signals via CD28 to the T cell are not sufficient to replace physiologic T-cell activation mediated by accessory cells.

To adapt the immunization protocol to the clinical situation, we designed a vaccination strategy with a defined dose of irradiated, proliferation-incompetent tumor cells (Figure 4). We also switched from the aggressively growing B16 melanoma model to the more moderate proliferating A20 lymphoma that is more similar to most courses of human cancer. However, in both tumor models a strong correlation was found between the generation of tumor-reactive antibodies after initial bsAb treatment and the survival of mice after rechallenge with tumor cells only.

A closer look at the induced humoral response revealed the presence of EpCAM-specific antibodies. The development of such antibodies was strictly restricted to mice vaccinated with intact bsAbs and was independent of an idiotypic network response (Table 3). To clarify that the immune protection was not based only on the artificially introduced human EpCAM antigen, we challenged the mice deliberately with untransfected A20 wild-type cells. In spite of this critical modification, all immunized mice survived as shown in Figure 5. Moreover, in the A20 B-cell lymphoma model we were able to raise an anti-Id response without

targeting the tumor-specific Id. These results provide clear evidence that our vaccination strategy yielded immune responses against various antigens of the individual tumor, independent of the surrogate target antigen EpCAM recognized by the bsAb on the tumor cell. The significant loss of immune protection using bsF(ab')$_2$ fragments of the identical bsAb underlines the importance of the Fc region in this process (Figure 5). Importantly, this finding was recognized prior to the wild-type challenge by the failure to detect tumor-reactive antibodies. Therefore, assessment of antitumor antibodies served as a prognostic factor for evaluating the success of the vaccination strategy (Table 2 and Figure 6).

By using bsAb and A20 wild-type cells without the target antigen EpCAM, we detected a humoral anti-A20 Id response, although the bsAb could not interact with A20 wild-type cells directly (Figure 6). This immune response is likely due to nonspecific activation of T cells and antigen-presenting cells induced by the trifunctional bsAb. However, the observed antibody response raised by this unspecific activation was rather weak. Moreover, challenging these mice revealed only a partial protection against the tumor (Figure 7). Therefore, optimal immunization and full antitumor protection was only accomplished when trifunctional bsAb and target antigen-expressing cells were combined.

Depletion experiments were used to evaluate the role of T cells in both the induction and the effector phase. As shown in Table 2, the induction of the tumor-specific humoral response was clearly diminished ($P < .0023$) after CD4$^+$ T-cell depletion in the priming phase. Moreover, a significant decrease of survival rate ($P = .02$) was observed in this group (Figure 9). These results indicated a clear contribution of a cellular immune response to the observed antitumor immunity. This finding could be further confirmed by the depletion of CD8$^+$ T cells in the effector phase, which caused a markedly loss of tumor protection. However, adoptive serum transfer experiments revealed only a weak protection by the isolated use of tumor-reactive antibodies (Figure 8). Taken together, humoral as well as cellular immune responses were generated against A20 lymphoma cells, whereby cell-mediated immunity may be of major importance.

Bendandi et al[32] recently described an anti-Id vaccination approach for follicular lymphoma in an adjuvant situation, resulting in molecular remissions. However, the encouraging results were hampered by the need to generate individual Id-producing hybridomas. Here, the use of intact bsAb in combination with sorted irradiated tumor cells could allow a simple application that is also capable of inducing an anti-Id response. Although we see no signs of toxicity in the preclinical mouse models, it will be essential to investigate possible side effects and toxicity of this potent immune activation in a clinical setting. In summary, the vaccination strategy described here achieves a long-lasting antitumor immunity without the complexity of gene transfer or cell-fusion techniques and therefore opens new perspectives for a broader clinical application.

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